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Calcitonin potential roles in the bonnethead shark, Sphyrna Tiburo

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CALCITONIN: POTENTIAL ROLES IN THE BONNETHEAD SHARK, *SPHYRNA TIBURO*

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CALCITONIN: POTENTIAL ROLE IN THE BONNETHEAD SHARK, *SPHYRNA TIBURO*

Stephanie M. Nichols

Calcitonin is a phylogenetically ancient hormone produced in the parafollicular C cells of the thyroid gland in mammals and the ultimobranchial gland in all other jawed vertebrates. Historically, this hormone was believed to be a major factor in calcium regulation. However, recent studies have indicated a potential role in reproduction and/or development. The current study presents data on serum calcitonin levels over the female reproductive cycle and calcitonin bioactivity during embryonic development of the bonnethead shark. Serum calcitonin concentrations within the mature female bonnethead shark were determined using an enzyme-linked immunosorbent assay (ELISA) for salmon calcitonin. Calcitonin bioactivity within the developing embryo was demonstrated via immunohistochemical techniques. Results from ELISA indicate an elevation in maternal serum calcitonin levels during the early stages of pregnancy. Calcitonin bioactivity was evident within the early pregnancy embryonic gastrointestinal tract and pancreas, suggesting a role in embryonic nutrition and development.

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INTRODUCTION

In 1962, Copp, Cameron, Chaney, Davidson, and Henze discovered the existence of a 'hypocalcemic factor' in mammals after infusion of the thyroid-parathyroid complex with a large dose of calcium resulted in reduction of systemic blood calcium. Hirsch, Gauthier, and Munson (1963) extracted this factor from rat and hog thyroid, and initially named it 'thyrocalcitonin' after its site of origin. Later studies (Foster et al., 1964; Bussolati & Pearse, 1967; Pearse & Carvalheira, 1967) confirmed the source of calcitonin from the thyroid, more specifically the parafollicular or C cells. The C cells arise embryologically from the terminal branchial pouch, which in lower vertebrates forms the ultimobranchial pouch. As the ultimobranchial pouch in lower vertebrates (birds, reptiles, fishes) was known to be homologous to the C cells in mammals, researchers subsequently confirmed the presence of calcitonin in the ultimobranchial gland (UBG) of lower vertebrates (O'Dor, Parkes, & Copp, 1969).

Calcitonin (CT) is a peptide hormone consisting of 32 amino acid residues. The composition of the amino acids coding for CT may vary considerably among the vertebrates. Based on primary structure and biological properties, the calcitonins sequenced thus far have been divided into four general lineages: cartilaginous, salmon, porcine, and human (Yoshida et al., 1997). Studies show that calcitonins of UBG origin exhibit a greater hypocalcemic effect in higher vertebrates than native calcitonin, possibly because of structural differences of the hormone between groups (Austin & Heath, 1981; Takei et al., 1991). In addition to the hormone's known production by the C cells of the

thyroid gland in mammals and the ultimobranchial gland (UBG) of all other jawed vertebrates, it may also be produced in extrathyroidal and extraultimobranchial sources.

The presence of the hormone calcitonin within all jawed vertebrates suggests the existence of a conserved physiological role among these animals. Numerous studies have attempted to define the physiological role of calcitonin. Initial studies focused on the hormone's involvement in calcium regulation and actions on the skeleton, then shifted to roles in other systems, such as the gastrointestinal and reproductive systems. To date the basic physiological role of CT in vertebrates remains controversial. Researchers have, therefore, long referred to CT as a "hormone in search of a function" (Austin & Heath, 1981).

Elasmobranchs (sharks, skates and rays) produce CT despite lack of bone and are the most phylogenetically primitive group to possess this hormonal system. The presence of CT in this group suggests it may be of fundamental importance as a vertebrate hormone. There is a limited number of studies on the physiological role calcitonin plays in elasmobranch fishes. In higher vertebrates, CT may mediate plasma calcium stasis mainly via inhibition of bone resorption. However, calcitonin immunoreactivity has been reported in animals that possess cartilaginous skeletons, indicating that the hormone may have a nonosseous function. Studies on the possible role of CT in extracellular calcium regulation of elasmobranchs have produced varied results. Hayslett et al. (1971) reported no effect of CT on plasma mineral concentrations in *S. acanthias*. In 1985, Glowacki, O'Sullivan, Miller, Wilkie, and Deftos demonstrated that CT induced a prompt hypercalcemia in the leopard shark (*Triakis semifasciata*). Ultimobranchialectomy

(UBX) in the stingray *Dasyatis akajei* significantly reduced plasma CT levels, however, did not affect plasma calcium levels (Suzuki, Takagi, Sasayama, & Kambegawa, 1995). A.K. Srivastav, S.K. Srivastav, Sasayama, and Suzuki (1998), also focusing on *D. akajei*, found that administration of salmon calcitonin produced hypocalcemia. If CT does play a role in calcium homeostasis, it is possibly via a mechanism different from that in higher vertebrates.

In contrast, evidence of a possible role for calcitonin in elasmobranch reproduction is accumulating. Takagi, Suzuki, Sasayama, and Kambegawa (1993) demonstrated that injections of estrogen stimulate calcitonin secretion in the stingray, *Dasyatis akajei*. Estrogen receptors have also been confirmed within the UBG of this species (Yamamoto, Suzuki, Takahashi, Sasayama, & Kikuyama, 1996). In 1998, Gelsleichter and Manire (unpublished data) observed fluctuations in serum CT concentrations of the mature female bonnethead shark (*Sphyrna tiburo*) from two regions off of southwest Florida, Tampa Bay and Florida Bay. The fluctuations appeared to be related to the reproductive cycle and were demonstrated using a sandwich enzyme-linked immunosorbent assay (ELISA) for salmon calcitonin. This particular assay was not validated or statistically demonstrated to accurately detect calcitonin in *S. tiburo* serum. In this placentally viviparous species, CT concentrations appeared to significantly elevate during the late pregnancy to post-partum stages. During late pregnancy in *S. tiburo*, embryos rely mainly on nutrition supplied directly from the maternal placenta. If this assay did accurately reflect serum calcitonin concentrations, elevations of serum calcitonin at this time could indicate a role in matrotrophy.

Although the basic physiological role of calcitonin in vertebrates has not been established, evidence demonstrates a likely involvement in the reproduction and/or development of vertebrates in general (a more complete description of the history of CT research in vertebrates is provided in Appendix I). Based on the results of studies by Takagi et al. (1993) and Yamamoto et al. (1996), which indicated CT secretion was induced by estrogen in elasmobranchs, and on the unvalidated results of Gelsleichter and Manire (unpublished) indicating a pattern in serum CT levels related to the reproductive cycle in *S. tiburo*, the current study was undertaken on *S. tiburo* to define the possible role of the hormone calcitonin in the reproduction and early embryonic development of this species. A brief description of the reproductive system morphology and reproduction in *S. tiburo* is provided below.

Sphyrna tiburo reproductive tract morphology

The reproductive tract of the female bonnethead shark (Figure 1) consists of paired ovaries and oviducts (Schlernitzauer & Gilbert, 1966). Only one of the ovaries is functional and the nonfunctional ovary usually atrophies or is reduced in size. The functional ovary supplies both oviducts with eggs.

The oviduct consists of four parts: the ostium into which eggs from the ovary are released; an oviducal gland (also known as the "shell" or "nidamental" gland) which stores sperm during the mating through pre-ovulation stages of reproduction and also secretes a thin membranous protective layer over the fertilized eggs; an isthmus or bridge from the oviducal gland to the uterine portion of the oviduct; and the uterus, where the

embryos develop until parturition. The posterior ends of the oviducts open into the cloaca through which the embryos will emerge.

Sphyrna tiburo reproduction

The bonnethead shark reproduces by placental viviparity. Bonnethead shark embryos initially rely on yolk stores, which are formed in the liver during vitellogenesis and stored in the oocytes prior to ovulation. Once these yolk stores are depleted and before placental development, the uterus begins secreting nutrients that may be ingested or absorbed by the growing embryos. The uterus has evolved several adaptations to ensure successful development of the growing embryos: enhanced secretory activity, expansion to accommodate the growing embryos, respiration, osmoregulation, waste disposal, and protection of the young (Hamlett, 1999). As gestation enters late stages, the yolk sac differentiates into a placenta and the uterus thins and increases in vascularity (Schlernitzauer & Gilbert, 1966). Certain areas of the uterus modify to form the utero-placental attachment sites across which nutrient and metabolic exchange will take place. The placental connection then "implants" into the uterine wall and embryos receive nourishment via the maternal placenta and also through continued uterine secretion. The bonnethead shark embryo also possesses appendiculae (finger-like projections) along the umbilical cord, which presumably increase surface area for nutrient, gas, and waste exchange.

Mating events in this species occur during early fall along with storage of sperm within the oviducal gland. Yolk production within the female begins in the fall in preparation for synchronous ovulation in early spring and resulting pregnancy. *S. tiburo*

reproduces with one of the shortest gestation periods (4.5 – 5 months) known in sharks (Parsons, 1993; Manire, Rasmussen, Hess, & Hueter, 1995). Parturition occurs in late summer and, after approximately 1 - 2 months, the reproductive cycle resumes (Figure 2).

Project Objectives

The specific objectives of the current study were to investigate: 1) the potential role of calcitonin in reproduction through analysis of serum calcitonin concentrations in the mature female bonnethead shark; 2) the bioactivity of calcitonin within the reproductively active tissues of female bonnethead sharks during all reproductive stages; and 3) the potential role of calcitonin in development through the localization of the hormone in embryonic tissues.

METHODS

Sample Collection

The bonnethead shark (*S. tiburo*) is a small hammerhead species of shark common in the coastal waters of the southeastern United States. Sampling for this species took place along the southwest coast of Florida in Tampa Bay (Figure 3) under an EPA-sponsored study conducted at the Mote Marine Laboratory in Sarasota, Florida. Mature female *S. tiburo* were collected using gill nets (mesh size 4-6''). Most samples were collected over a year starting in March 1997 and ending in February 1998, with additional collections made during years 1999-2000. This schedule ensured that samples of these females covered all the reproductive stages from mating to post-partum events. Specimens were measured, weighed, and sexed. Blood samples were collected via caudal venipuncture and returned to the laboratory on ice. After the blood was allowed to clot for 3-6 hours (to facilitate serum/plasma separation), blood was centrifuged for 5 minutes at 5,000 rpm and the serum separated and frozen at -20°C. Serum was later thawed and calcitonin concentration in the samples were measured using ELISA.

Mature female specimens were returned to the laboratory and examined both internally and externally to determine the reproductive stage according to the characteristics described by Manire et al. (1995) for this species. These parameters included evidence indicating: external mating wounds; sperm presence in the oviducal gland; size of ovarian follicles; presence of uterine ova, embryos, and placenta.

Multiple samples of various tissues were collected from individual sharks and preserved in 10% formalin prepared using phosphate buffered saline modified for use

with elasmobranch tissues for 48-72 houts, then transferred to 70% ethanol for storage. The archived tissue bank was later used to examine specific tissues of interest for calcitonin immunoreactivity. The samples taken included: reproductive tissues (uterus, oviducal gland, ovary, liver) from the stages of reproduction in which they were active. Based on the earlier observation by Gelsleichter and Manire (unpublished) on serum CT activity over the reproductive cycle of *S. tiburo* peaking at the late stages of reproduction, the uterus was sampled over the entire reproductive cycle (mating, preovulation, ovulation, postovulation, early pregnancy, implantation, late pregnancy, post partum) to demonstrate the changes in development and assumed CT bioactivity within this tissue.

Samples were also collected from embryos including: appendiculae, gills, thyroid, spleen, kidney, interrenal gland, stomach, duodenum, pancreas, and intestine from the embryo. Ultimobranchial tissue from both adult and embryo bonnethead sharks was also sampled. Due to the morphology and topography of this gland within the pericardial cavity, it was very difficult to pinpoint its location in this species. However, the gland was finally located following serial sectioning of the pericardial wall. A goal was to obtain tissue samples from at least five individual sharks for each period of interest. In early pregnancy embryos (those measuring less than 10 mm total length), cross sections at various points along the specimen's body were taken to allow for a reliable visualization of a number of organs without the loss of the small tissues within the animal. In larger embryos, individual tissues were dissected.

Antibodies Used in Assays

A polyclonal antibody to salmon calcitonin (sCT) that had been raised in rabbit was obtained from Peninsula Laboratories (Belmont, CA). Past studies have traditionally used salmon CT in elasmobranch studies successfully as it is 60-70% structurally similar to elasmobranch CT (Suzuki, Ueda, Sakamoto, & Sasayama, 1999a).

In an attempt to improve the sensitivity of these experiments, synthetic dasyatid CT (dCT) was also employed because it is group-specific. Based on the published sequence structure of the Takei et al. (1991) report for *Dasyatis akajei*, a synthetic dasyatid peptide was produced by Alpha Diagnostic International Incorporated (San Antonio, TX). This peptide was injected into two rabbits over a period of three months to produce polyclonal antisera to synthetic dCT.

ELISA

Two types of ELISA were utilized over the course of the project. The first, an indirect ELISA, was determined to be an unreliable method for detection of calcitonin in the female bonnethead shark using dCT and dCT antisera. A more sensitive ELISA, a competitive inhibition ELISA using dCT and dCT antisera (CI-ELISA 1), was developed following failure of the indirect ELISA. This assay also did not reliably detect CT levels in the serum of the mature female bonnethead shark using dCT. However, competitive inhibition ELISA using sCT and sCT antisera (CI-ELISA 2) was validated as a reliable detector of the hormone in the serum of the mature female bonnethead shark and eventually used to characterize seasonal changes in female bonnethead shark serum CT

concentration.

Principle Behind the Indirect ELISA

In the indirect ELISA, calcitonin in the standard or unknown (serum) sample is initially adsorbed to the walls of polysterene microtiter plates as the solid-phase antigen. Following overnight incubation of the coated plate, the wells are incubated with a blocking solution to prevent any remaining absorption sites from reacting. The wells are then incubated with an antibody directed against the solid-phase antigen. Following a washing step to remove unbound antibody, the wells are incubated with antispecies antibody conjugated to an enzyme. The wells are then incubated with a substrate which will react with the enzyme to produce a color reaction. An acidic stopping solution is added and the degree of enzymatic turnover of the substrate is determined by dual wavelength absorbance measurement at 630 and 450 nm. Using this method, the absorbance measured is directly proportional to the concentration of calcitonin present. A set of CT standards is used to plot a standard curve of absorbance versus CT concentration from which the calcitonin concentrations in the serum can be estimated.

Principle Behind the Competitive Inhibition ELISA

In the competitive inhibition ELISA, a known amount of calcitonin is initially adsorbed to the wells of polystyrene microtiter plates as the solid-phase antigen. In separate glass tubes, standards or unknown (serum) samples (liquid-phase antigen) are

preincubated with antibody to calcitonin overnight at 4°C. Following the overnight incubations of the liquid-phase antigen/antibody solution, the coated plates, which have undergone exposure to a blocking solution, are filled with the incubation mixture. The solid-phase antigen and liquid-phase antigen compete for calcitonin antibody binding sites. The unreacted antibody in the solution binds with the solid-phase antigen with proportionately less reacting as the amounts of liquid-phase antigen increases. Following this incubation, the wells are washed and incubated with an antispecies antibody conjugated to an enzyme. This solution will react with the existing solid-phase antigen-antibody complexes. The plates are washed and incubated with a color-producing substrate. An acidic stopping solution is added and the degree of enzymatic turnover of the substrate is determined by dual wavelength absorbance measurement at 630 and 450 nm. Using this method, the amount of color developed is inversely proportional to the amount of original liquid-phase antigen present in the antigen-antibody solution. A set of calcitonin standards is used to plot a standard curve of absorbance versus calcitonin concentrations from which the hormone concentration in the unknown (serum) samples can be calculated.

Indirect ELISA Procedure

Antigen coating

Calcitonin (dCT) was diluted in carbonate/bicarbonate coating buffer (0.05M, pH 9.6) and administered at 100 µl/well. Indirect ELISA required coating with standard dilutions of dCT (0, 0.2, 0.3, 0.4, 0.6, 0.8, 1.2, 1.6, 2.4, 3.2, 4.8, 6.4, 9.6, 12.8, 19.2, 25.6, 38.4,

51.2, and 76.8 ng/mL) to establish an appropriate range of standards with which to determine serum CT concentrations. The transfer into the wells was done in less than 2 seconds per well to avoid adsorption of protein to the plastic tip of the pipette. Plates were covered and incubated overnight at 4°C. After incubation, the coating buffer was aspirated from the wells using pipettes and the plate washed three times with phosphate buffered saline (8.77 g NaCl, 1.2 g NaH2PO4, 1 liter of filtered water, pH 7.0-7.2) containing 0.05% Tween 20 (PBST) to remove all unbound antigen. All wells were then incubated with 200 µl of a blocking solution (PBST with 2% nonfat milk) for 2 hours at room temperature to reduce non-specific binding of the antibody and followed by three washes of PBST.

Primary antibody incubation

Following washing, all wells were incubated with primary antibody at a dilution of 1/10,000 dCT, which was considered ideal following an antibody titer dilution test of 1/500, 1/1,000, 1/5,000, 1/10,000, and 1/50,000. The primary antibody was delivered at 100 µl/well and incubated for 2 hours at room temperature. This was followed by aspiration and multiple rinses before incubation with the labeled secondary antibody.

Labeled secondary antibody incubation

Following rinsing, all wells received 100 µl of goat antirabbit IgG conjugated to horseradish-peroxidase (HRP) (Sigma, St. Louis, MO) diluted at 1:2000 in PBST. Plates

were incubated at room temperature for 45 minutes, then aspirated and washed three times with PBST.

Peroxidase activity determination

To detect horseradish peroxidase activity, all wells received 100 µl of tetramethylbenzidine (TMB) substrate solution (Sigma, St. Louis, MO). Color development proceeded for 30 minutes at room temperature and in the dark because TMB is light sensitive. At 15 minutes into the development, absorbance of light was measured at 630 nm using a microplate reader. At the end of 30 minutes, the reaction was terminated with the quick addition of an acidic stopping solution (2 M sulfuric acid) at 100µl/well and a final absorbance reading was taken at 450 nm.

Competitive Inhibition ELISA Procedure

Two competitive inhibition ELISAs were evaluated for use in measuring serum CT concentration in female bonnethead shark serum. The first (CI-ELISA 1) used dCT and dCT antisera, whereas the second (CI-ELISA 2) used sCT and sCT antisera. A brief summary of the competitive ELISA procedure is described below and the full protocol is provided in Appendix II.

Antigen coating

In both CI-ELISA 1 and 2, the solid-phase antigen was diluted in carbonate/bicarbonate coating buffer (0.05M, pH 9.6) and administered at 100µl/well.

CI-ELISA 1 used a standard dCT coating of 25 ng/mL based on the previous indirect ELISA standard titer curve. In CI-ELISA 2, wells were coated with serial dilutions of purified sCT (1.6, 2.4, 3.2, 4.8, 6.4, 9.6, 12.8, 19.2, 25.6, 38.4, 51.2, and 76.8 ng/mL) to establish an ideal coating concentration of 50 ng/mL. This concentration was determined to be ideal because it resulted in a maximum absorbance reading within the range of the microplate reader (0 – 2 absorbance units) (Bio-Rad Model 550). In both cases, the transfer into the wells was done in less than 2 seconds per well to avoid adsorption of protein to the plastic tip of the pipette. Plates were covered and incubated overnight at 4°C. After the incubation period, the coating buffer was aspirated from the wells using pipettes and the plate washed three times with PBST to remove all unbound antigen. All wells were then incubated with 200 µl of a blocking solution (PBST with 2% nonfat milk) for 2 hours at room temperature to reduce non-specific binding of calcitonin antibodies, which was followed by three washes of PBST.

Preparation of standards and samples

In both competitive ELISAs, the blocking step was followed by an addition of the liquid-phase antigen/antibody solution. The standards and unknown (serum) samples were prepared by dilution in assay buffer (PBST with 1% nonfat milk). In individual glass tubes, standards and unknowns were incubated overnight at 4°C with antibody also diluted in assay buffer. Competitive inhibition ELISA (CI-ELISA 1 and 2) proceeded using standard liquid-phase antigen dilutions of 0, 0.1, 0.2, 0.39, 0.78, 1.56, 3.13, 6.25, 12.5, 25, 50, and 100 ng/mL. An optimal standard dilution for CT-ELISA 2 was

established at 50 ng/mL as a result of the validation portion of the ELISA (See "RESULTS - Competitive Inhibition ELISA").

Antibody dilution for CI-ELISA 1 was made at 1/10,000 based on the indirect ELISA. An antibody titer dilution test for CI-ELISA 2 established an ideal dilution of 1/25,000 following experimentation with dilutions 1/10,000, 1/20,000, 1/40,000, and 1/80,000. Following incubation, the solutions were dispersed in triplicate (standard) and duplicate (serum samples) at 100 µl/well into wells that had been previously coated and blocked. The wells were incubated at room temperature for 2 hours, then aspirated and washed three times with PBST.

Non-specific binding of the antibody to proteins other than calcitonin was determined by incubating serum for 24 hours at 4°C with Norit-A charcoal (Sigma Chemical Co., St. Louis, MO) (0.05 g charcoal/1mL plasma), a substance that will bind 50 ng CT/g charcoal (Robertson, 1981), before use in ELISA experiments. The response of these samples was then compared to the response of samples that had not been treated. Changes in absorption after treatment were due to non-CT antibody activity.

Labeled secondary antibody incubation

Following rinsing, all wells received 100 µl of goat antirabbit IgG conjugated to HRP (Sigma, St. Louis, MO) diluted at 1:2000 in PBST. Plates were incubated at room temperature for 45 minutes, then aspirated and washed three times with PBST.

Peroxidase activity determination

To detect HRP activity, wells received 100 µl of TMB solution (Sigma, St. Louis, MO). Color development proceeded in the dark for 30 minutes at room temperature. At 15 minutes into the development, absorbance was measured at 630 nm using a microplate reader (Bio-Rad Model 550). At the end of 30 minutes, the reaction was stopped with the quick addition of 2 M sulfuric acid at 100 µl/well and a final absorbance reading was taken at 450 nm.

Validation of Immunoassay

Absorbance measurements of the standard samples and unknown (serum) samples were plotted against dilution of pooled serum samples. When it appeared that the standards and unknowns behaved in a similar manner upon visual inspection (i.e., the produced curves appeared similar or parallel), statistical demonstration of a similar dose-response relationship between the curves was necessary to validate the assay system. Using the linear equation derived from the standard log absorbance versus log dilution curve, the amount of calcitonin detected in the diluted serum samples was determined and potency estimates for these samples were established. Potency estimate, which was determined by dividing absorbance by dilution factor, represents the amount of CT present in the total serum sample. Following a method for statistical analysis of similarity established by Diamandis and Christopoulos (1996), analysis of variance (ANOVA) was used to determine if potency estimate was independent of the dilution of serum samples assayed. In all statistical tests, the alpha level was set at 0.05. A linear regression was

also performed to test if the potency estimate was independent of the dilution of the serum samples analyzed. This method of analysis is preferable as it is more rigorous than ANOVA in this situation (Diamandis & Christopoulos, 1996). A graph of potency estimate versus dose was constructed and a t test was performed to determine if the observed slope was different from zero.

Determination of Serum Calcitonin Concentrations over the Reproductive Cycle

Once the immunoassay was validated, actual calcitonin concentrations in the serum samples were estimated using the linear equation established by the standard curve (log absorbance versus log dilution). Following determination of the average serum calcitonin concentrations over the different stages of the reproductive cycle, ANOVA was performed to test for differences. When variances did not meet the homogeneity assumption, non-parametric tests were used to demonstrate whether or not the medians were significantly different. Although the data set required non-parametric testing as it did not conform with ANOVA assumption of equal variances, means ± SEM were still reported for comparison with other studies.

Histology

Tissue sections were embedded in paraffin wax (Fisher Scientific, Fair Lawn, NJ) following immersion in a graded series of alcohols (70 – 100%) and limonene-based clearing agent (Hemo-De, Fisher Scientific, Pittsburgh, PA). Embedded tissue was

sectioned (5 μm) using a rotary microtome. The sections were adhered to microscope slides coated with Poly-L-Lysine, and dried at 37°C for 24 hours.

Slides from each specimen's tissue sample were stained using Harris' Hematoxylin and Eosin (HHE) to visualize general cellular structure throughout tissue and also to ensure that the sections made were a good representation of the given tissue. HHE is one of the most commonly used techniques in animal histology and routine pathology. Hematoxylin is a basic dye that stains acidic structures, such as nuclei, a purplish blue. In contrast, eosin is an acidic dye which stains basic structures, such as cytoplasm, red or pink (Wheater, Burkitt, & Daniels, 1987).

Slides containing tissue samples that had been embedded in paraffin were deparaffinized and rehydrated in a graded series of EtOH (100%, 100%, 95%, 95%). Following a running tap water bath, the slides were incubated in Harris' Hematoxylin as the primary staining agent. The samples were then run through another water bath and acid alcohol bath to remove excess hematoxylin. Following another water bath, a strong basic solution of sodium bicarbonate was used to shift the color of the hematoxylin in the sections to blue. This step was followed by incubation in the eosin counterstain, dehydration in a graded series of EtOH (95%, 95%, 100%, 100%), and immersion in a clearing agent (Hemo-De). Following incubation in the clearing agent, the slides were coverslipped with Cytoseal-60 (Stephens Scientific, Kalamazoo, MI). The full protocol for this staining procedure is provided in Appendix II.

Immunohistochemistry

Following examination of general cellular structure for a given tissue sample, the presence of immunoreactive calcitonin (ir-CT) was determined using polyclonal antisera against salmon and/or synthetic dasyatid CT. The appropriate antibody titer for immunohistochemistry (IHC) was tested using various concentrations of the antibody (1/500, 1/1,000, 1/5,000, 1/10,000, 1/25,000, 1/50,000) and chosen based on the quality of the stain in positive controls. Anti-salmon calcitonin was subsequently utilized for the majority of the IHC experiments because of the superior quality and clarity of the stain using this antisera. The Avidin-Biotin complex (ABC) method was used to visualize the location of the primary antibody interaction with ir-CT. Tissue sections were processed using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). A brief review of the immunohistochemical staining procedure is described below. The full protocol is contained in Appendix II.

Tissue sections were deparaffinized in Hemo-De (Fisher Scientific, Pittsburgh, PA) and rehydrated in a descending series of alcohols followed by a running tap water rinse. Before treatment with antisera, tissue sections were exposed to an antigen retrieval solution (10mM sodium citrate, pH 6.0) at 95°C for 30 minutes and allowed to cool for 1 hour afterward. This technique was used to "unmask" antigens that may have been covered during fixation. The tissues were rinsed briefly in PBS and incubated overnight in a blocking solution, which consisted of 2% normal goat serum. Following this step, slides were rinsed in PBS and blocked for endogenous biotin activity using an Avidin/Biotin Blocking kit (Vector Laboratories, Burlingame, CA). This step ensured

that all endogenous biotin, biotin receptors, or avidin binding sites that may have been present in the tissue would not react inappropriately with the primary antisera.

The slides were rinsed with PBS and incubated for 24 – 96 hours with the diluted primary antibody (1/10,000 dCT; 1/25,000 sCT). Following this incubation, slides were rinsed with PBS and incubated with diluted biotinylated goat anti-rabbit IgG for 30 minutes. The slides were then rinsed in PBS and incubated with a hydrogen peroxide/methanol solution (1:1) for 30 minutes to block endogenous peroxidase activity. After this, the slides were rinsed with PBS and incubated with ABC solution, which contains an Avidin DH - biotinylated horseradish peroxidase H reagents complex, for 45 minutes. This was followed by a PBS rinse and application of diaminobenzidine hydrochloride (DAB) for a maximum of 8 minutes to produce a reddish-brown stain where immunoreactive CT was present. After a 5 minute running tap water rinse, the slides were counterstained in a 2% methyl green solution (Vector Laboratories, Burlingame, CA) for one hour. This was followed by a rinse in tap water, dehydration in a series of graded alcohols, incubation in clearing agent, and coverslipping using Cytoseal-60.

Controls for specificity of reaction

Controls for specificity of the reaction were performed through the: 1) validation of the IHC process via deletion of one step at a time to ensure no inappropriate reaction took place with an important component of the process missing; 2) use of sections of ultimobranchial gland to act as the positive control; 3) replacement of the primary

antiserum with dilutant (G-PBS) to act as a negative control; and 4) addition of another negative control using pre-absorbed primary antisera. Pre-absorbed antisera was prepared using the following procedure: purified CT was prepared to a concentration of 5000 ng/mL in carbonate/bicarbonate coating buffer then applied to a microwell plate (100 μl/well) and incubated overnight at 4°C. Following the incubation, the plate was washed three times with PBST, then incubated with a blocking solution (PBST with 2% nonfat milk) for 2 hours. After washing with PBST three times, the plate was incubated with diluted primary antisera for 2 hours. Following this, the resultant solution (presumably containing no antibody as it should have been bound to the plate) was aspirated and applied to tissue sections. When pre-absorbed antibody negative controls were prepared, normal antibody was prepared following the same protocol except the plate was not coated with antigen, only with the coating buffer alone.

RESULTS

Enzyme-linked immunosorbent assay (ELISA)

Indirect ELISA

Various dilutions of polyclonal antibody to dasyatid calcitonin were tested against serial dilutions of synthetic dasyatid calcitonin standard to obtain the titer curve (Figure 4). A Vmax, represented by the inflection point of the curve, of 1/10,000 dilution was obtained for the antibody (log 4 = 10,000). This dilution was maintained in the assay.

Serial dilutions of dCT ranging from 1.6 to 76.8 ng/mL were coated to a plate and followed by incubation with the 1/10,000 antibody concentration. Calcitonin coating at 51.2 ng/mL resulted in the maximum absorbance unit reading of approximately 1.6 after a 30 minute color development (Figure 5), which was within the range of the plate reader (0 – 2 optical density (OD) units). Because of this, a coating of 50 ng/mL was used in the subsequent ELISAs.

An indirect ELISA using dasyatid calcitonin to demonstrate cross-reactivity of female *Sphyrna tiburo* serum with the calcitonin antiserum was subsequently performed after establishing ideal coating and antibody conditions (Figure 6). The failure of this assay to demonstrate a similar or parallel relationship between the standard curve and unknown (serum) absorbances at serial dilutions prompted the evaluation of a competitive inhibition ELISA.

Competitive Inhibition ELISA

The competitive ELISA for dasyatid calcitonin (CI-ELISA 1) was not validated as an accurate assay for serum calcitonin concentrations in the mature female bonnethead shark. A parallel relationship was not demonstrated between the standard curve and the unknown (serum) curves at various dilutions (Figure 7).

A competitive ELISA using salmon calcitonin (CI-ELISA 2) was developed. A calcitonin coating of approximately 50 ng/mL and antibody dilution of 1/20,000 resulted in a maximum absorbance value of approximately 1.4, which was in the range of the plate reader (Figure 8). The subsequent competitive ELISAs for salmon calcitonin used 50 ng/mL sCT to coat and an antibody dilution of 1/25,000 (for a slight increase in sensitivity).

The analysis of CI-ELISA 2 demonstrated the cross-reactivity of anti-salmon antibody with various concentrations of pooled female bonnethead shark serum (Figure 9A, B). Upon visual inspection, the linearized standard curve and serum curve appeared to have a similar or parallel relationship. ANOVA was peformed to test similarity under the working null hypothesis that the curves were not different (i.e., potency estimates at different levels of serum dilution were not significantly different). As the variances were not homogeneous, a non-parametric test (Kruskal-Wallis one-way ANOVA) was performed. This resulted in a non-significant F ($F = 0.2019$), supporting the null hypothesis that the potency estimate of the unknown was independent of dose and was, therefore, similar to the standard. A linear regression was also performed on potency estimate versus amount of sample analyzed. This resulted in a significant F of 0.0072.

When two outliers from the group of replicate measurements of serum levels at dose 0.05 mL were removed to eliminate the effect of these anomalies, the resulting F equaled 0.0957, which was non-significant. This result supported the earlier ANOVA in demonstrating that the unknown and standard analyte reacted in a similar manner.

Calcitonin concentrations in mature female bonnethead shark serum measured over the reproductive cycle of specimens collected in Tampa Bay demonstrated elevations in serum CT concentrations at post-ovulation and a peak at early pregnancy before dropping off (Figure 10). ANOVA resulted in a significant difference in serum CT concentrations between reproductive stages. As the variance homogeneity assumption was not met, non-parametric Kruskal-Wallis one-way ANOVA was performed. The resulting F was significant (F = .0162). Following this result, Mann-Whitney U tests were performed to identify stages that were significantly different. Significant differences ($\alpha = 0.05$) were observed between early pregnancy and mating (P = 0.0253), preovulation (P = 0.0275), and post partum (P = 0.0472) stages.

Immunohistochemistry

Antibody Validation

The use of polyclonal antisera to salmon calcitonin was suitable for immunolocalization of CT in elasmobranch tissues as demonstrated by IHC visualization using bonnethead shark ultimobranchial gland. The general structure of the UBG consisted of highly vascularized tissue with many follicles (Figure 11A). These follicles were lined with parenchymal cuboidal epithelium and contained granular material in the

follicular cavities. No ir-CT was detected in either negative control (G-PBS control and pre-absorbed antibody control) (Figures 11B and C).

Strong immunoreactivity was seen, as expected, in the positively treated section of UBG (Figure 11D). The staining in this gland was limited to parenchymal secretory cells that line the follicular cavity.

Histology and Immunohistochemical Detection of CT in Maternal Tissues

Reproductive System

Uterus

The uterus underwent several modifications (Figure 12) to accommodate the developing embryo over the reproductive stages: mating (A, B); preovulation (C, D); ovulation (E, F); postovulation (G, H); early pregnancy (I, J); late pregnancy (K, L); and post partum (M, N). Since the original study by Gelsleichter and Manire (unpublished) demonstrated serum CT concentrations peaking at a stage when the uterus is the dominantly active reproductive organ, the uterus was focused on over all reproductive stages. As in other placental sharks, the uterus of *S. tiburo* was composed of simple columnar epithelium lined with secretory cells overlaying a vascularized bed with crypts and invaginations. As pregnancy proceeded, the uterus retained its general structure, but increased the number of crypts and invaginations. Following parturition, the surface epithelium remained intact and remodeled for the next cycle. No evidence of ir-CT was demonstrated in the uterine components (including muscle and connective tissue) over the reproductive cycle.

Oviducal Gland

The general structure of the oviducal gland of the bonnethead shark (mating shown, Figure 13A) consisted of several secretory follicles lined with cuboidal epithelial cells. The gland also contained several sperm-storing follicles (not shown). No ir-CT was present in the epithelial cells of the secretory vesicles or in the sperm-storing follicles of the oviducal gland during the early stages of the reproductive cycle when sperm storage is occurring (Figure 13B).

Liver

The general structure of the liver (preovulation shown, Figure 13C) demonstrated a highly vascularized tissue with pigment (melanocytes) occurring in patches throughout (Andrew & Hickman, 1974). Cuboidal or polyhedral hepatic parenchymal cells, which are very high in fat and glycogen content, were scattered throughout the tissue. No ir-CT was present in the liver during the early phases of the reproductive cycle (Figure 13D) when vitellogenesis, production of egg nutrients, is taking place.

Ovary

Throughout the ovulatory cycle the ovarian follicles containing the oocytes (Figure 13E) appeared to grow continuously. The follicles contained oocytes at different stages of development and those about to break out of the ovary and enter the oviduct were located near the periphery closest to the ostium. The follicles were embedded within a loose, connective tissue. Immunoreactive CT was not present in the ovary during the

early phases of the reproductive cycle (Figure 13F) when follicular development is occurring.

Histology and IHC of Embryonic Tissues

A variety of embryonic tissues from both early and late pregnancy embryos were surveyed for evidence of CT bioactivity (Summary in Table 1). Immunoreactive CT was observed in the gastrointestinal tract and pancreas of the early pregnancy embryo.

Ultimobranchial Gland

The ultimobranchial gland was located within the early pregnancy embryo via serial sectioning of the pericardial wall. The morphology and topography of the gland was identical to the adult UBG. Follicles were evident lined with epithelial cells surrounding a lumen containing a granular matrix (Figure 14A). Interestingly, no calcitonin bioactivity was seen (Figure 14B) in the embryo UBG at this early stage of development. Also evident in these samples were sections of cartilage in which no ir-CT was evident.

Sites of Gas Exchange

The appendiculae (Figure 15A), finger-like projections along the umbilical cord of the embryo, developed as early as when the embryo grew to 3 mm total length (Nichols, personal observation, April 2001). The umbilical cord contained both an artery and a vein surrounded by a connective tissue sheath. The villiform extensions along the umbilical cord were lined with columnar epithelial cells and appeared to grow

continuously into late pregnancy. No ir-CT was present in the appendiculae (Figure 15B) of the bonnethead shark embryo.

Early on in the development of the embryo, gills were external and protruded into the uterine fluid medium. As development proceeded, the internal gills formed. Both external and internal gills were focused on in the experiments and produced similar results. Gills at a later stage of pregnancy, therefore internal, were demonstrated. Each individual gill filament was covered with lamellae (Figure 15C). Individual lamella consisted of a double sheet of epithelial cells overlying a basement membrane containing blood channels. No ir-CT was present in the gills of the early and late pregnancy embryo (Figure 15D).

Thyroid

The thyroid was made up of lobular follicles (Figure 16A). Each follicle was lined with epithelial cells ranging from cuboidal to columnar. These cells contained nuclei near the basement membrane. The lumen of the follicles contained a colloid substance secreted by the epithelial cells. No ir-CT was evident in the thyroid of the early and late pregnancy embryo (Figure 16B).

Immune Tissues

The spleen was a highly vascularized organ . The tissue was made up of white pulp containing plasma cells, lymphocytes, and granulocytes and red pulp containing erythrocytes (Figure 17A). The whole tissue was infused with large blood vessels and

capillaries, which drain into the hepatic portal vein (Hamlett, 1999). No ir-CT was present in the spleen of the embryo (Figure 17B).

Renal/Interrenal Complex

The kidney and interrenal tissues were found in close association within the bonnethead shark (Figure 18A). The section shown represented a zone of kidney tissue referred to as "bundle zone" (Hamlett, 1999). This portion was made up of tightly packed tubules surrounding the interrenal tissue. The interrenal tissue appeared to be of one cell type. Immunohistochemical stain of the kidney-interrenal section (Figure 18B) did not demonstrate evidence of ir-CT in either tissue of the early and late pregnancy embryo.

Gastro-Entero-Pancreatic System

Stomach

Cross section of the early pregnancy stomach demonstrated simple columnar epithelial cell mucosa layer lining the lumen of the stomach and overlying connective tissue and smooth muscle (Figure 19A). No ir-CT was present in the stomach of the embryo (Figure 19B).

Duodenum

Cross section of the duodenum demonstrated a structure similar to that of the stomach, with the mucosa layer being much wider in this anterior portion of the intestine (Figure 19C). Immunoreactive CT was present in this portion of the gastrointestinal tract

of an early embryo bonnethead shark. Staining was limited to the mucosa cells of the epithelial lining (Figure 19D) and appeared to have a general distribution pattern among these cells from the base of the mucosa layer to the lumenal edge.

Pancreas

The pancreas of the bonnethead shark was a double lobed gland surrounded by a sheath of connective tissue lying in close proximity to the stomach. The pancreas was made up of two tissue types – exocrine and endocrine. A large percentage of pancreas consisted of alveoli (exocrine ducts) (Figure 19E). The exocrine ducts were surrounded by lymphoid and hemopoietc (small round cells) tissue. Each alveoli contained large pyramidal cells surrounding a central lumen into which the cells secrete digestive enzymes. Immunoreactive CT was demonstrated within the pancreas of the early pregnancy embryo (Figure 19F) and appeared evident in both the exocrine and endocrine tissue of the samples examined.

Intestine

The folds of the intestine were lined with columnar epithelial cells overlying smooth muscle (Figure 19G). There were no cilia along the length of the epithelial lining because of the developed muscular layers. No ir-CT was seen in the intestine of the embryo (Figure 19H).

DISCUSSION

Sphyrna tiburo reproduces by placental viviparity, a process in which young are initially nourished by a large yolk supply and uterine secretions until late pregnancy when a maternal/fetal exchange organ (placenta) develops (Manire et al., 1995). Results from the present study indicate that calcitonin may play a role in this advanced mode of reproduction, especially during the early pregnancy stage, when a peak in serum calcitonin concentration of mature female bonnethead sharks was evident. The developed competitive inhibition ELISA using salmon calcitonin and antisera to salmon calcitonin is the first to demonstrate a validated pattern of serum calcitonin concentrations in an elasmobranch during all stages of reproduction. Although there was no immunohistochemical staining within reproductive tissues, demonstration of calcitonin bioactivity within the gastrointestinal tract and pancreas of the early pregnancy bonnethead shark embryo suggests that the observed increase in maternal serum calcitonin levels may exert a role in early embryonic nutrition.

The lack of immunoreactive calcitonin within the reproductive tissues of the mature female bonnethead shark was surprising, especially given previous studies in mammalian models, which have demonstrated calcitonin binding and production in these tissues (Zhu, M.K. Baghhi, & I.C. Bagchi, 1998). As there was no indication of calcitonin bioactivity within the reproductive tissues of the female bonnethead shark, the hormone may be reflected instead in uterine fluid content and not recognizable using IHC methodology. Future work would include examination of uterine fluid content of the bonnethead shark for the presence of the hormone. An alternative explanation for the

lack of immunoreactive calcitonin may be that the bonnethead shark uterus produces a calcitonin that is structurally different from ultimobranchial calcitonin and not recognizable by the antibodies used.

Calcitonin may not directly affect reproductive apparati and instead exert effects on non-reproductive tissues yet to be determined. For example, calcitonin has been shown to be involved in gill function in many lower vertebrates (Milhaud, Rankin, Bolis, & Benson, 1977; Milet , Peignoux-Deville, & Martelly, 1979; Arlot-Bonnemains, Fouchereau-Peron, Moukhtar, & Milhaud, 1983; Martial et al., 1994). In the Atlantic stingray (*Dasyatis sabina*), calcitonin bioactivity has been demonstrated in gill tissue (Gelsleichter, personal communication, August 2001) and may act to reduce the flux of calcium and other nutrients across the gill to maintain a stable internal environment. Preliminary immunohistochemical staining of gill tissue of adult *S. tiburo*, however, does not indicate that is the case for this species (Gelsleichter, pers. comm.).

If the maternal serum calcitonin target during early pregnancy is the developing embryo, the hormone may play a substantial role in gastrointestinal activity and nutrient absorption as calcitonin immunoreactivity within the duodenum and pancreas of the early pregnancy embryo provide evidence for this potential role. These portions of the gastrointestinal system are responsible not only for digestive processes, but also initial nutrient absorption. Earlier studies on calcitonin and gastrointestinal activity have demonstrated a relationship between the hormone and this system. In particular, calcitonin bioactivity within the duodenum and pancreas of humans, dogs, and rats influences exocrine pancreatic enzyme activity, secretory volume in response to ingested

material and bicarbonate secretion (Goebell, 1976; Hotz & Goebell, 1981; Nakashima, Appert, & Howard, 1977; Tanaka et al., 1989). Calcitonin has also been shown to have an inhibitory effect on insulin secretion and indirectly increase glucagon secretion (Giugliano, Passariello, Sgambato, Torella, & D'Onofrio, 1982; Giugliano, 1984; Caviezel & Mangili, 1983; Alwmark, Stavinoha, Cooper, Greeley Jr., & Thompson, 1986). These hormones, insulin and glucagon, behave antagonistically in the control of blood glucose levels. These findings certainly suggest a role for calcitonin in the regulation of digestive processes, possibly indicating an involvement in nutrient uptake as ingested material is broken down into usable forms and the initial stages of absorption begin.

A limited number of studies is available in demonstrating the relationship of calcitonin and gastrointestinal activity within lower vertebrates. However, there is evidence of calcitonin bioactivity within the gastrointestinal tract for several teleost species (Roos, Bundy, Bailey, & Defots, 1974; Sasayama , Abe, Suzuki, & Hayakawa, 1996; Okuda, Sasayama, Suzuki, Kambegawa, & Srivastav, 1999; Suzuki et al., 1999b) and one elasmobranch species, the spiny dogfish (*Squalus acanthias*) (El-Salhy, 1984), suggesting a potential involvement in nutrient regulation and/or absorption.

If maternal calcitonin exerts a role in embryonic nutrition, it would have to be able to permeate the surrounding egg capsule. While the early pregnancy embryo is yolk-dependent at this stage of development, transfer of maternal compounds does occur at this time provided the compounds are able to penetrate. Although little data are available concerning egg capsule permeability in elasmobranchs, a study by King (T. King and T.

Koob, personal communication, July 2001) suggests calcitonin is within the range of egg capsule permeability and is likely to transfer. To confirm this possibility, future work would further test the limits of permeability for the bonnethead shark egg capsule.

Alternatively, if immunoreactive calcitonin demonstrated in the early pregnancy embryo gastrointestinal tract and pancreas is not maternally derived, calcitonin may be produced in the embryonic gastrointestinal tract itself. The source would appear to be extraultimobranchial as the UBG of the early pregnancy embryo was not active, a discovery indicated through immunohistochemical staining.

Immunohistochemical techniques can only indicate presence or absence of calcitonin within a given tissue. Attempts to clarify whether a certain tissue was binding or producing calcitonin were performed using the immunogold technique. This method is designed to identify specific binding sites for calcitonin within a given tissue. Immunogold involves exposure of tissue to biotinylated calcitonin, which presumably binds the receptor specific for the hormone, followed by an incubation with an anti-biotin gold conjugate for visualization. Unfortunately, this technique consistently produced very high background staining after several attempts and, therefore, unusable results.

Future studies would attempt to define calcitonin's presence in these tissues by utilizing alternative techniques, such as targeting calcitonin mRNA expression within given tissues to clarify sites of production. Once the nature of calcitonin presence within the duodenum and pancreas of the early pregnancy embryo is elucidated, that is, whether calcitonin is binding and/or being synthesized in these tissues, specific studies can be conducted to examine the functional relationship between calcitonin and the various

gastrointestinal/pancreatic hormones and enzymes using both *in vivo* and *in vitro* dosage experimentation.

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APPENDIX I - HISTORY OF CALCITONIN RESEARCH IN VERTEBRATES

Mammals

Extracellular Calcium Regulation

Extracellular calcium is tightly controlled because of its role in the activity of excitable tissues such as nerves and muscle. Perturbations in the amount of extracellular calcium can have dramatic effects on a number of important physiological processes including: neuromuscular excitability, muscle contraction, activity of enzymes, permeability of cell membranes, and intracellular metabolic events (Copp et al., 1970). Total extracellular calcium represents less than 0.1% of total body calcium; intracellular calcium making up 0.5% and the skeleton 99.4%. The amount of calcium in the skeleton serves as a large reservoir, which provides support while retaining an easily accessible supply of calcium (Wendelaar Bonga & Pang, 1991).

Early studies of calcitonin focused on its potential role in calcium regulation because of its ability to cause reductions in plasma calcium levels. Along with parathyroid hormone (PTH), which functions to resorb calcium from skeletal reserves and raise plasma calcium levels, CT was considered a major factor in the regulation of extracellular calcium. The hormone's main function was defined as inhibition of calcium resorption from bone in antagonistic action to PTH.

In young mammals, birds and reptiles, the effects of CT have been clear and undisputed (Copp & Klein, 1989). Administration of CT in these young animals results in a fall in plasma calcium levels. However, administration of CT in adults frequently

does not reduce plasma calcium levels. In addition, removal of calcitonin-excreting tissue is not lethal and experimental animals are still able to maintain internal calcium homeostasis (Austin & Heath, 1981). Therefore, this hormone does not appear to be essential for the routine maintenance of serologic mineral stasis. Since endogenous calcitonin secretion will prevent or decrease hypercalcemia, the role of this hormone may be to correct excessive calcium levels, a response that has been described as 'antihypercalcemic'. (Talmage, Grubb, Norimatsu, & Vanderwiel, 1980; Wendelaar Bonga & Pang, 1991).

Actions on Skeleton

One of the best described actions of CT is its ability to suppress osteoclast (resorptive cell) activity at high doses. Calcitonin decreases both the amount and activity of osteoclast ruffled borders, the metabolically active sites in bone resorption (Talmage et al., 1980), thereby protecting the skeleton from demineralization. This action is especially important during periods of increased calcium demand, such as pregnancy, lactation, and early development. During these periods, increased physiological demand for calcium is usually met by through enhanced absorption of calcium in the gastrointestinal tract. This action is accomplished via increased plasma levels of the active hormonal form of vitamin D (1,25[OH]2D3), which is also a bone resorptive hormone. An increase in CT secretion has also been documented at this time (Stevenson, Hillyard, & MacIntyre, 1979; Stevenson et al., 1983) and may indicate that calcitonin acts in opposition of the bone resorptive action of 1,25[OH]2D3 and, thereby, protects the

skeleton from demineralization. CT may act in conservation of ingested calcium via storage of calcium in the fluid bathing bone surfaces (Talmage et al., 1980). This temporary storage of calcium may be used between calcium intake intervals and, therefore, combat bone-resorptive activities and conserve skeletal reserves.

In addition to CT's ability to inhibit bone mineral resorption, there is evidence that CT may also aid skeletal development by promoting mineralization. Wase, Solweski, Rickes, and Seidenberg (1967) reported an increase in corticol bone growth in rats and rabbit that had been treated with calcitonin. A later study by Boris, Hurley, Trmal, Mallon, and Matuszewski (1979) demonstrated that administration of salmon calcitonin to rats that had been exposed to a mineralization-blocking agent showed significantly improved mineralization of the tibial epiphyseal plate. Burch and Corda (1985) found that calcitonin stimulated growth of mammalian growth plate cartilage of the young rat *in vivo* and the fetal pig *in vitro*. Studies have shown that serum calcitonin levels are elevated in premature and newborn human infants and significantly decline after the first year of life (Klein, Wadlington, Collins, Catherwood, & Deftos, 1984). Since individuals undergo the maximal rate of bone growth at this time, elevations in serum CT levels may support a role in bone growth and/or mineralization.

Calcitonin's action on the skeleton lends itself to human biomedical application. This hormone has been used to treat individuals suffering from degenerative bone diseases such as Paget's disease. This metabolic bone disorder is characterized by an abnormally high number of osteoclasts, leading to structurally weakened bones (Hadley, 1996). CT, in this case, will bind to the osteoclasts and disenable them, therefore, preventing further

skeletal denigration. Another clinical use of calcitonin has been in the treatment of osteoporosis. Many females experience progressive loss of bone density and thinning of bone tissue as they age concurrent with a decrease in gonadal steroids, in particular, estrogen (Hadley, 1996). Structural variants of salmon calcitonin supplements are typically prescribed as therapeutic agents to preserve bone intergrity since these calcitonins exert a greater hypocalcemic effect than mammalian calcitonin.

Gastrointestinal Function

Calcitonin has been shown to act in an antihypercalcemic function during dietary calcium uptake. Therefore, it is of interest that factors originating from the gastrointestinal system have been reported to potentially influence CT secretion. Following a meal, ingested material undergoes the process of digestion in the gastrointestinal tract (GIT), which involves a timely and well-coordinated interplay of various hormones and enzymes. Upon entering the GIT, ingested material stimulates the production of gastrin, a hormone produced by the gastrin cells of the pyloric mucosa that induces the secretion of gastric juices containing pepsin, renin and hydrochloric acid (Holmes, 1979; Hale & Margham, 1988). Cooper, Schwesinger, Mahgoub, and Ontjes (1971) found that low doses of gastrin infused into pig thyroid stimulated calcitonin secretion. Plasma gastrin levels rise postprandially (during and after a meal) and the peptide is reported to induce hypocalcemia (Wendelaar Bonga & Pang, 1991). As CT secretion is stimulated by gastrin, the hypocalcemic action of the gastrin may be mediated by calcitonin. However, in adult men and rats, a significant gastrin – calcitonin

relationship has not been established. A rise in plasma gastrin levels of the rat and human following a meal did not coincide with a rise in plasma calcitonin levels and attempts to stimulate calcitonin secretion via injections of gastrin were unsuccessful (Wendelaar Bonga & Pang, 1991).

The initial stages of digestion will produce an acid chyme, which will induce secretion of secretin by the duodenum, the anterior portion of the intestine. This hormone will induce the secretion of bile from the liver and pancreatic juice from the exocrine pancreas (Norris, 1997). These substances contain enzymes, enzyme-precursors, and salts to break down the ingested material and bicarbonates to buffer the acid chyme. The hormone secretin has been reported to influence calcitonin secretion in human and rat subjects (Sethi, Kukreja, Bowser, Hargis, & Williams, 1981). Elevation of plasma calcitonin concurrent with secretin secretion may indicate an involvement in nutrient uptake as food material is broken down into storable forms and/or a role in buffering. It is also possible that CT acts to control the amount of pancreatic juice secreted in response to secretin as CT has been demonstrated to significantly inhibit exocrine pancreas activities, namely reducing pancreatic enzyme secretions in man (Goebell, 1976; Hotz & Goebell, 1981). Studies have also demonstrated that CT significantly inhibits not only enzyme output, but also secretory volume and bicarbonate secretion in man, cats and dogs (Nakashima et al., 1977; Tanaka et al., 1989).

Specialized cells, termed islets of Langerhans, in the endocrine pancreas secrete hormones such as glucagon and insulin into the blood stream in response to digestive material passing through the gastrointestinal tract. These hormones behave

antagonistically to control blood sugar levels. Insulin acts to reduce blood sugar levels by stimulating the absorption of glucose into muscle and adipose tissue and by stimulating the conversion of glucose into glycogen and fats. Glucagon causes the breakdown of glycogen and release of glucose into the blood. Giugliano et al. (1982) and Giugliano (1984) evaluated the effect of calcitonin on insulin and glucagon responses to glucose introduced intravenously in man. In a dose-dependent manner, calcitonin was shown to have an inhibitory effect on insulin secretion and also reduces the inhibitory effect glucose has on glucagon secretion. Caviezel and Mangili (1983) confirmed CT suppressor activity on insulin secretion via endogenous CT elicited by calcium administration. Isolated rat pancreatic islets, which were induced to secrete insulin, were also inhibited by CT *in vitro*, providing support for CT inhibitory activity on insulin production taking place via direct action on islet tissue (Alwmark et al., 1986). The physiological importance of CT's effect on insulin and glucagon secretion has not been established. However, current findings do suggest a role for CT in the regulation of digestive processes.

Nervous System

Immunoreactive calcitonin and calcitonin gene-related peptide, which is encoded by from the calcitonin gene (Evans, 1993), have been localized in the nervous system. The peptides may function as neurotransmitters and neuromodulators rather than hormones in these areas (Kaneko, Harvey, Kline, & Pang, 1989; Sasayama, Katoh, Oguro, Kamgebawa, & Yoshizawa, 1991). However, calcitonin produced in the central nervous

system has not been shown to represent a significant contribution to the total amounts of calcitonin in the general circulation.

Reproduction and Development

Recent evidence suggests a role for calcitonin in mammalian reproduction and/or development. Plasma calcitonin concentrations are high in pregnant and lactating females (Stevenson et al., 1979; Klein, et al., 1984). During periods of reproduction and parental care, large amounts of calcium are required to supply extra energy and nutrients to the developing embryo. Due to calcitonin's ability to suppress osteoclast function, it has been proposed that the hormone may act to protect the maternal skeleton from resorption (Stevenson et al., 1979; Klein et al., 1984; Kovacs & Kronenberg, 1997) during this period of elevated stress on maternal calcium reserves.

Estrogen, a major ovarian hormone active during reproduction, acts directly on bone and also indirectly via action on the parathyroid and C cells, to regulate production of CT and PTH. Estrogen has been demonstrated to lead to an increase in C cell size and number (Stevenson et al., 1983). Studies on rats that had undergone oophorectomy (removal of the ovaries, an estrogen secreting tissue) show a decrease in plasma calcitonin levels in response to calcium stimulus. The effects of the oophorectomy in the rat can be reversed by administration of estradiol (Catherwood, Onishi, & Deftos, 1983). Greenberg et al. (1986) found that administration of both estrogen and progesterone (another major reproductive ovarian hormone) was effective in stimulating CT secretion. Naveh-Many, Almogi, Livni, and Silver (1992) also demonstrated that the parathyroid

and C cells contain estrogen receptors and administration of 17ß-estradiol to oophorectomized rats led to a fourfold increase in calcitonin mRNA levels.

Recently, Ding, Zhu, M.K. Bagchi, and I.C. Bagchi (1994) and Zhu et al. (1998) have demonstrated that expression of CT within mammals is particularly enhanced during the period of implantation, the "receptive phase" of pregnancy when the embryo is implanted into the uterine wall. The glandular epithelial cells of the uterus have been shown to be a site of increased CT production during implantation (Kumar et al., 1998; Wang, Rout, Bagchi, & Armant, 1998). Calcitonin is secreted into the uterine lumen immediately before implantation of the blastocyst into the uterine wall takes place. Zhu et al. (1998) demonstrated that suppression of CT production via disruption of the basic genetic process reduced embryonic implantation in rats by as much as 80%. The ability to suppress CT production was made possible through antisense sequencing, in which artificially constructed DNA sequences were designed to bind to RNA and attract cleavage enzymes to the RNA to snip it up, therefore, disenabling CT synthesis. A blockage of embryonic implantation in rats due to CT suppression indicates CT may be essential for this crucial phase of reproduction to occur.

Plasma CT concentrations are also elevated in premature and newborn infants (Klein et al., 1984). Large amounts of calcium are also required during periods of rapid growth such as embryonic development and the first year of life. Since plasma calcitonin levels are greatly elevated during these periods, it has been proposed that in addition to protecting the maternal skeleton from resorption, calcitonin may act to divert calcium to the embryo for skeletal development.

Nonmammalian Bony Vertebrates

Extracellular Calcium Regulation

Evidence for the role of calcitonin in calcium regulation within bony non-mammalian vertebrates is not well-supported. Studies on the effect of CT on plasma calcium levels have obtained conflicting results. Lopez, Peignoux-Deville, Lallier, Martelly, and Milet (1976) reported that administration of CT to the eel *Anguilla anguilla* results in hypocalcemia. A study on sticklebacks (*Gasterosteus aculeatus*) produced the same results (Wendelaar Bonga, 1981). However, Fouchereau-Peron, Arlot-Bonnemains, Moukhtar, and Milhaud (1987) reported that CT induced hypercalcemia in grey mullet and rainbow trout (both fresh and sea-water adapted). This was also observed in female brown trout (*Salmo trutta*) that had been administered sCT intraperitoneally (Oughterson et al., 1995). More often, CT administration has had a lack of effect on plasma calcium levels. For example, CT administration in either freshwater catfish (*Heteropneustes fossilis*: Singh & Srivastav, 1980), Japanese eels (*Anguilla japonica*: Hirano, 1981) or Atlantic cod (*Gadus morhua*: Bjornsson, 1985) did not affect plasma calcium levels.

Control of calcium homeostasis and metabolism is quite different between terrestrial and aquatic vertebrates, which may explain the differences in CT activity between these animals. In particular, parathyroid glands are not present in the lower vertebrates, making an appearance only in terrestrial animals. In the case of aquatic vertebrates, plasma calcium levels are directly affected by calcium concentrations in their environment because of the intimate contact between blood and water through the skin and gill

surfaces. Lower vertebrates may possess unique hormones in the regulation of extracellular calcium. For example, stanniocalcin, which is produced by the corpuscles of Stannius in fish, appears to play a role in preventing hypercalcemia and prolactin, which is produced in the pituitary gland, appears to play a role in calcium metabolism by exerting a hypercalcemic effect (Wendelaar Bonga & Pang, 1991). In fishes, CT receptors have been located in the gill, a major organ responsible for nutrient and gas exchange in these animals (Arlot-Bonnemains et al., 1983). Milhaud et al. (1977) and Milet et al. (1979) have reported that CT decreases calcium influx and enhances calcium efflux out of the gills in fishes exposed to calcium-rich water. A more recent report by Martial et al. (1994) demonstrates not only the presence of CT receptors in the gill tissue of the salmonid *Oncorhynchus gorbuscha*, but also the expression of an encoded CT gene in the gills suggesting an autocrine and/or paracrine function of CT in the gill. This organ appears be an important target for calcitonin despite the lack of effects on plasma calcium.

Pressures to maintain extracellular calcium homeostasis vary among the different vertebrate groups coinciding with a shift from an aquatic and calcium-rich environment to a terrestrial existence where the main supply of calcium is through the diet. Studies on both aquatic and terrestrial amphibians demonstrate a hypocalcemic effect resulting from CT administration (Krishna & Swarup, 1985; Srivastav & Rani, 1989). However, in some species of aquatic amphibians, the function of CT may be comparable to that of terrestrial animals – a role in suppressing hypercalcemia. Ultimobranchialectomy (UBX) in both the frog *Rana pipiens* (Robertson, 1970) and bullfrog tadpoles (Sasayama &

Oguro, 1976) results in hypercalcemia upon exposure to a calcium rich environment that can be reduced upon transplantation of ultimobranchial tissue or salmon calcitonin injection (Robertson, 1970). These animals have evolved specialized compartments to store calcium (Robertson, 1971; Yoshida et al., 1997) and CT's role may be to promote storage in these sacs, which serve as important mineral reservoirs for such activities as metamorphosis. The effect of CT on the calcium homeostasis between and among these groups varies greatly, making it difficult to define the role of this hormone in this physiological action.

Actions on Skeleton

There are limited studies on the effects of calcitonin on skeletal protection and formation in nonmammalian vertebrates. These studies generally support a role for CT in bone mineralization and protection of skeletal reserves. For example, Lopez et al. (1976) demonstrated that sCT administration increases the degree of mineralization in immature eels (*Anguilla anguilla)* and will inhibit bone demineralization in mature eels. In mature females of the same species, UBX results in significant demineralization of the bone matrix.

Many lower vertebrates have evolved acellular bone (bone that does not contain enclosed osteocytes). Wendelaar Bonga and Lammers (1982) reported that injections of sCT into tilapia, *Sarcotherodon mossambicus*, a fish with acellular bone, did result in appositional bone growth, osteoblast activation, and scale formation, although action was very slow. Although there was some evidence to support skeletal action, there were no

noticeable effects on plasma calcium levels or the mineral concentration in bone which may have been because *S. mossambicus* has a skeleton with no osteocytes and little or no bone-resorbing cells. Thus, CT's action in calcium exchange between bone and plasma in animals with acellular bone would presumably be limited.

As in mammals, it has been argued that the action of calcitonin on the skeleton of non-mammalian jawed vertebrates may be particularly important when bone growth is rapid or skeletal reserves are at risk, for example, during early development, female sexual maturation and rapid ovarian development (Bjornsson, Haux, Forlin, & Deftos, 1986; Evans, 1993).

Gastrointestinal Function

Although there are limited studies involving the relationship between calcitonin and gastrointestinal processes in the lower vertebrates, there is some support that gastrointestinal hormones can influence CT activity. Roos et al. (1974) found that supraphysiological doses of pancreozymin, a hormone produced in the duodenum that stimulates secretion of pancreatic juices, induced CT secretion in the cultured trout. In addition, immunocytochemical investigation of the GIT demonstrated the presence of immunoreactive calcitonin in each of the 4 compartments making up the intestinal tract of the goldfish (Okuda et al., 1999).

In an attempt to clarify the role of CT in GIT activity, Suzuki et al. (1999b) induced hypercalcemia in freshwater eels (*Anguilla japonica*) through diet and measured serum CT levels. An increase in plasma CT levels was demonstrated with a postprandial rise of

plasma calcium concentrations. Sasayama et al.(1996) reported a significant difference in plasma calcitonin levels between calcium-fed goldfish and those serving as the control, although the differences were not statistically significant. Since previous studies demonstrate that calcium infused directly into the bloodstream does not influence CT concentration (Shiraki, Hasegawa, Hirano, & Orimo, 1982), the studies by Suzuki et al. (1999b) and Sasayama et al. (1996) suggest that CT secretion in fish may be influenced by a combination of calcium and nutrient absorption by the intestine. The nature of the nutrients involved has not been documented. Although it appears CT may play a role in GIT functioning, further studies to define this role in teleosts and other vertebrates have yet to be completed.

Reproduction and Development

Evidence supporting a role for calcitonin in reproduction has been demonstrated in most bony nonmammalian vertebrate groups (Norberg et al., 1989). Increased UBG activity and calcitonin binding are observed during female sexual maturation in a number of teleosts, birds, and reptiles (Bjornsson et al., 1986; Norris, 1997; Bentley, 1998). Norris (1997) observed seasonal changes associated with the reproductive cycle in the UBG activity of some reptiles, and changes in UBG development and calcitonin activity have also been associated with the reproductive process in egg-laying hens (Bentley, 1998).

Serum CT levels significantly increase in *Anguilla japonica* (H. Yamauchi, Orimo, K. Yamauchi, Takano, & Takahashi, 1978), *Salmo gairdneri* (now *Oncorhynchus mykiss*:

Bjornsson et al., 1986), and *Salmo trutta* (Norberg et al., 1989) during female sexual maturation while no change in serum CT levels has been observed in males. Increase in plasma calcitonin levels during sexual maturation in the female teleost occurs prior to ovulation, a period of rapid gonadal growth which involves the production of calcium-rich phospholipoproteins (vitellogenins) in the liver and subsequent transport of these proteins to the ovaries. It is unclear if high calcitonin secretion during female gonadal maturation, which is under the control of estrogens (Scott & Sumpter, 1983), is linked to skeletal protection against demineralization as in mammals.

APPENDIX II – METHOD PROTOCOLS

General Procedure for Competitive Inhibition ELISA using Salmon Calcitonin

DAY ONE:

Prepare coating buffer - dissolve carbonate/bicarbonate tablet in 100mL filtered water.

1) Coat plate with 50 ng/mL of standard salmon calcitonin (sCT) (100 µl in each well)

To Make:
- starting with 10µl aliquot (of 1mg/mL sCT) add 990 µl coating buffer to bring to initial solution - 10,000 ng/mL

- mix 990 µl coating buffer with 10µl (10,000 ng/mL)
  - 100 ng/mL

- mix 1 mL coating buffer with 1 mL (100 ng/mL)
  - 50 ng/mL

After coating preparation and addition of 100 µl to each well, cover with parafilm and plastic lid. Incubate overnight in 4°C.

On this day, also prepare liquid-phase antigen/antibody mixture (the "competitive" portion of the ELISA)

1) Prepare blocking dilutant - PBS-T with 1% nonfat dry milk

2) Prepare antibody dilution of 1/12,500 (Goal is 1/25,000, prep the dilution as double of what is desired, when combine with liquid-phase antigen portion of the solution, it will halve)

- using an sCT antibody (Ab)starting point dilution of 1/1,000, mix 80 µl of sCT Ab with 920 µl PBS-T with milk – result is antibody dilution of 1/12,500

3) Prepare standards:

- starting with 1 mg/mL (10 µl aliquot, add 990 PBS-T with milk to get 10,000 ng/mL)
- to 100 µl (10,000 ng/mL) add 900 µl PBS-T with milk - 1000ng/mL
- PBS-T with milk = "dil"

Standards wanted:	**Actually make:**	**To Make:**
100 ng/mL	200 ng/mL	mix 200 µl (1000ng/mL) + 800 µl dil.
50 ng/mL	100 ng/mL	250 µl (200 ng/mL) + 250 µl dil
25 ng/mL	50 ng/mL	250 µl (100 ng/mL) + 250 µl dil
12.5 ng/mL	25 ng/mL	250 µl (50 ng/mL) + 250 µl dil
6.25 ng/mL	12.5 ng/mL	250 µl (25 ng/mL) + 250 µl dil
3.13 ng/mL	6.25 ng/mL	250 µl (12.5 ng/mL) + 250 µl dil
1.56 ng/mL	3.13 ng/mL	250 µl (6.25 ng/mL) + 250 µl dil
0.78 ng/mL	1.56 ng/mL	250 µl (3.13 ng/mL) + 250 µl dil
0.39 ng/mL	0.78 ng/mL	250 µl (1.56 ng/mL) + 250 µl dil
0	0	

Combine liquid-phase antigen and antibody in glass tubes:

100 ng/mL - 200 µl of 200 ng/ml + 200 µl of 1/12,500 Ab - get 400 µl of 100 ng/ml with 1:50K

50 ng/mL - 200 µl of 100 ng/ml + 200 µl of 1/12,500 Ab - get 400 µl of 50 ng/ml with 1:50K

and so on...

for **0** - 200 µl of PBS-T with milk + 200 µl of 1/12,500 Ab

4) Serum samples -

For a given sample, each well will be incubated with 100 µl of Ab/serum mixture.
Desired series of serum dilutions: 50 parts serum, 40 parts, 30, 20, 10.
For each individual well -

50 parts serum - well will contain 50 µl Ab and 50 µl serum
40 parts serum - well will contain 50 µl Ab + 40 µl serum/10 µl dil
30 parts serum - well will contain 50 µl Ab + 30 µl serum/20 µl dil
20 parts serum - well will contain 50 µl Ab + 20 µl serum/30 µl dil
10 parts serum - well will contain 50 µl Ab + 10 µl serum/40 µl dil

Cover both standard/antibody and serum/antibody mixtures with parafilm and plate cover and incubate in 4°C overnight.

DAY TWO:

Remove coated plate and PBS -T out of fridge and allow them to reach room temperature.

Prepare blocking solution: PBS-T + 2% nonfat dry milk

1) Aspirate solution out of wells carefully (avoiding contact with bottom and sides of well)

2) Rinse wells with 200 µl PBS-T (three times, aspirating after each individual wash)

3) Tap wells over towel to get rid of remaining moisture following final PBS-T wash

4) Block remaining absorption sites with 200 µl blocking solution for 2 hours at room temperature

5) Aspirate dry and wash three times with 200 µl PBS-T

6) Add mixtures (Ab/standards, Ab/serum) according to planned plate layout at 100 µl per well, and incubate for 2 hours at room temp

7) Aspirate dry and wash three times with 200 µl PBS-T

8) Add diluted HRP-conjugated goat anti-rabbit IgG (100 µl per well) and incubate for 45 minutes at room temp

- to make: want 1:2000, take 5 µl (AR-IG) and add to 10 ml PBS-T

9) Aspirate dry and wash three times with 200 µl PBS-T

10) Add 100 µl TMB (or OPD) solution for 30 minutes (measure absorption at 15 minutes and 630 nm). Make sure plate is covered during this time as the subsrate is light sensitive

11) Quickly add 100 µl of stopping solution (2M H2SO4) to change color, measure at 450 nm.

Note: If running ELISA with a sample serving as a zero calcitonin control - you will use Norit-A (Sigma Chemical Company, St. Louis, MO) charcoal to bind up calcitonin within the sample. This sample will need to be prepped the day before Day One. Add 0.025 g charcoal to 500 µl serum, incubate in refrigerator overnight before centrifuging and aspirating out the "clean" serum and adding to Ab for further incubation.

Harris' Hematoxylin and Eosin Staining Procedure
(As taken from the Virginia Institute of Marine Science Histology Department)

	Step	
Deparaffinize	1	Clearing Agent (Hemo-De) - 5 minutes
	2	Clearing Agent (Hemo-De) - 5 minutes
	3	100% EtOH - 1 minute
Hydrate	4	100% EtOH - 1 minute
to	5	95% EtOH - 1 minute
Water	6	95% EtOH - 1 minute
	7	Running tap water wash - 3 minutes
	8	Harris Hematoxylin - 4 minutes
Stain	9	Running tap water wash - 4 minutes
	10	Acid Alcohol (0.3 ml in 250 ml 70%EtOH) - 2 minutes
Differentiate Hematoxylin	11	Running tap water wash - 5 minutes
	12	NaHCO3 (Sodium bicarbonate), saturated - 2 minutes
"Blue"	13	Running tap water wash - 3 minutes
Counterstain	14	Eosin Y - 3 minutes
	15	95% EtOH - 5 seconds
Differentiate Eosin &	16	95% EtOH - 5 seconds
Dehydrate	17	100% EtOH - 10 seconds
	18	100% EtOH - 10 seconds
	19	Clearing Agent (Hemo-De) - 3 minutes
Clear	20	Clearing Agent (Hemo-De) - 5 minutes

Immunohistochemistry Procedure

Note: Paraffin sections should be dried at 37-45°C for at least 1 day prior to immunostaining procedure.

Day 1

Note: Dilutants, distilled water (DW) and non-sterile phosphate buffered saline (N-PBS), should be at room temperature.

1) Set temperature on Isotemp water bath to 95°C. Fill white polypropylene Coplin staining jar with antigen retrieval solution (10mM sodium citrate, pH 6.0) and place in the water bath with the top loosely attached. Allow water bath and antigen retrieval solution to reach 95°C.
2) Deparaffinize and hydrate slides with clearing agent, graded alcohol (100-95%), and water. Use steps 1-7 from the HHE staining sequence. Once slides have been through the running tap water rinse (step 7) for 5 minutes, they can remain in tap water (not running) until the antigen retrieval solution/water bath reaches 95°C.
3) Once the water bath reaches the set temperature, place slides in the antigen retrieval solution and incubate for 30 minutes.
4) After 30 minutes, remove the entire Coplin jar(s) from the water bath and allow it to cool at room temperature for 1 hour.
5) Once the antigen retrieval solution has cooled, remove the slides and incubate in DW for 5 minutes at room temperature.
6) Prepare blocking serum during this time. The appropriate blocking serum should be from the animal in which the second antibody was prepared (goat for calcitonin in this case). Normal goat serum (NGS) is prepared by adding 3 drops of stock solution (Vectastain Elite ABC kit) to 10 mL of PBS in appropriate dropper bottle. Keep NGS out at room temperature until it is used.
7) Remove slides and incubate in PBS for 5 minutes at room temperature.
8) Remove slides and shake briskly to remove fluid. Use an oil-based pen to encircle individual sections that will be stained and place in immunostaining dish. The preserve moisture in the samples, dish should contain a paper towel moisetened with DW. Use 1-2 sections from each slide as a negative control. Do not allow time for sections to dry.
9) Carefully fully cover all sections (including negative control) with NGS. Once all the sections are covered, carefully cover the immunostaining dish and incubate in 4°C overnight.

Day 2

1) Remove immunostaining dish, PBS, and G-PBS out of the refrigerator and allow them to reach room temperature (~ 1 hour)

2) Remove/discard NGS and rinse slides in PBS 3X for 5 minutes at room temperature. The first PBS wash should contain 1 drop of Tween 20 detergent.

3) Remove slides and briskly shake to remove fluid. Cover sections with Avidin solution and incubate in the covered immunostaining dish for 15 minutes at room temperature.

4) Remove/discard fluid and rinse slides in PBS (no Tween 20) once for 5 minutes at room temperature.

5) Remove slides and briskly shake to remove fluid. Cover sections with Biotin solution and incubate in the covered immunostaining dish for 15 minutes at room temperature.

6) Remove/discard fluid and rinse in PBS 3X for 5 minutes at room temperature. The first wash contains 1 drop of Tween 20.

7) Remove slides and briskly shake to remove fluid. Cover sections with diluted primary antibody and incubate them in the covered immunostaining dish for 1 hour at room temperature. **Cover the first negative control with G-PBS instead of primary antibody and the second negative control with pretreated antibody instead of primary antibody. This is the only difference between the negative controls and all other sections.** Afterwards, incubate at 4°C for 24-96 hours.

Day 3

1) Remove immunostaining dish, PBS and DW from 4°C and allow them to reach room temperature (~ 1 hour). Leave the dish out 1 hour longer for each day that the sections were incubated in primary antibody.

2) Remove/discard/save primary antibody and rinse slides in PBS 3X for 5 minutes each at room temperature. The first rinse should contain 1 drop of Tween 20. Used primary antibody can be saved in a labeled container in the refrigerator and re-used if necessary.

3) Prepare fresh diluted biotinylated second antibody solution in the appropriate dropper bottle by adding 1 drop of stock solution biotinylated antibody and 3 drops of stock solution normal serum (Vectastain Elite ABC kit) to 10 mL of PBS.

4) Remove slides from rinse and shake briskly to remove fluid. Cover sections with diluted biotinylated antibody solution and incubate in the covered immunostaining dish for 30 minutes at room temperature. During this time, prepare fresh endogenous peroxidase blocking solution by mixing 2 mL of 3% hydrogen peroxide with 2 mL of methanol in a small graduated cylinder. Also prepare fresh ABC solution by adding 1 drop of solutions A and B each (Vectastain Elite ABC kit) to 5 mL of PBS in the appropriate dropper bottle.

5) Remove/discard fluid and rinse slides in PBS 3X for 5 minutes at room temperature (No Tween 20).

6) Remove slides from the rinse and shake to remove fluid. Cover sections with the hydrogen peroxide/methanol solution and incubate in the covered immunostaining dish for 30 minutes at room temperature.

7) Remove/discard fluid and rinse slides in PBS 3X for 5 minutes each at room temperature (No Tween 20).

8) Remove slides from the rinse and shake briskly to remove fluid. Cover sections with ABC solution and incubate in covered immunostaining dish for 45 minutes. During this time, filter Methyl Green solution and place the covered staining dish in the Isotemp water bath set at 37°C.

9) Remove/discard fluid and rinse slides in PBS 3X for 5 minutes each at room temperature (No Tween 20). During this time, prepare fresh DAB solution in a clean dropper bottle by adding 2 drops of buffer solution (Vector Labs DAB substrate kit) to 5 mL of DW (Not PBS) and mixing well, adding 4 drops of DAB stock solution and mixing well, then adding 2 drops of hydrogen peroxide (from the kit) solution and mixing well.

10) Remove slides from wash and cover sections with DAB staining solution. Incubate in non-covered immunostaining dish for 30seconds - 8 minutes. Watch negative control and terminate reaction in all slides if any nonspecific staining occurs.

11) Rinse slides in running tap water rinse for 5 minutes.

12) Remove slides from water rinse and incubate in the covered Methyl Green solution for 1 hour at 37°C.

13) Rinse slides in tap water for no more than 2 minutes total time. Exchange water only until appears as a light green and no further.

14) After the 2 minute tap water rinse, remove slides and dehydrate them by placing

them in graded alcohols (95%, 95%, 100%,100%). Slides should be dipped in the first three solutions (95% - 1, 95% - 2, 100% - 3) for 5 seconds each, then placed in final solution (100% - 4) for 10 seconds. Afterwards, place the slides into the first clearing agent for 5 minutes, then into the final clearing agent for 5 or more minutes. Slides can be stored in this solution until they are mounted.

15) Mount slides using Cytoseal mounting media and dry them overnight on a slide warmer at 37°C.

Table 1. Summary of embryonic tissue survey for calcitonin bioactivity.

Embryo Tissue	Stage of Development	Calcitonin Bioactivity (- absent, + present)
Ultimobranchial Gland	early pregnancy	-
Appendiculae	early pregnancy	-
	late pregnancy	-
Gills (external)	early pregnancy	-
Gills (internal)	late pregnancy	-
Thyroid	early pregnancy	-
	late pregnancy	-
Spleen	early pregnancy	-
	late pregnancy	-
Kidney-Interrenal	early pregnancy	-
	late pregnancy	-
Stomach	early pregnancy	-
	late pregnancy	-
Pancreas	early pregnancy	+
	late pregnancy	-
Duodenum	early pregnancy	+
	late pregnancy	-
Intestine	early pregnancy	-
	late pregnancy	-

Figure 1. General schematic of the reproductive tract of the female shark with one ovary demonstrated (adapted from Springer & Gold, 1989).

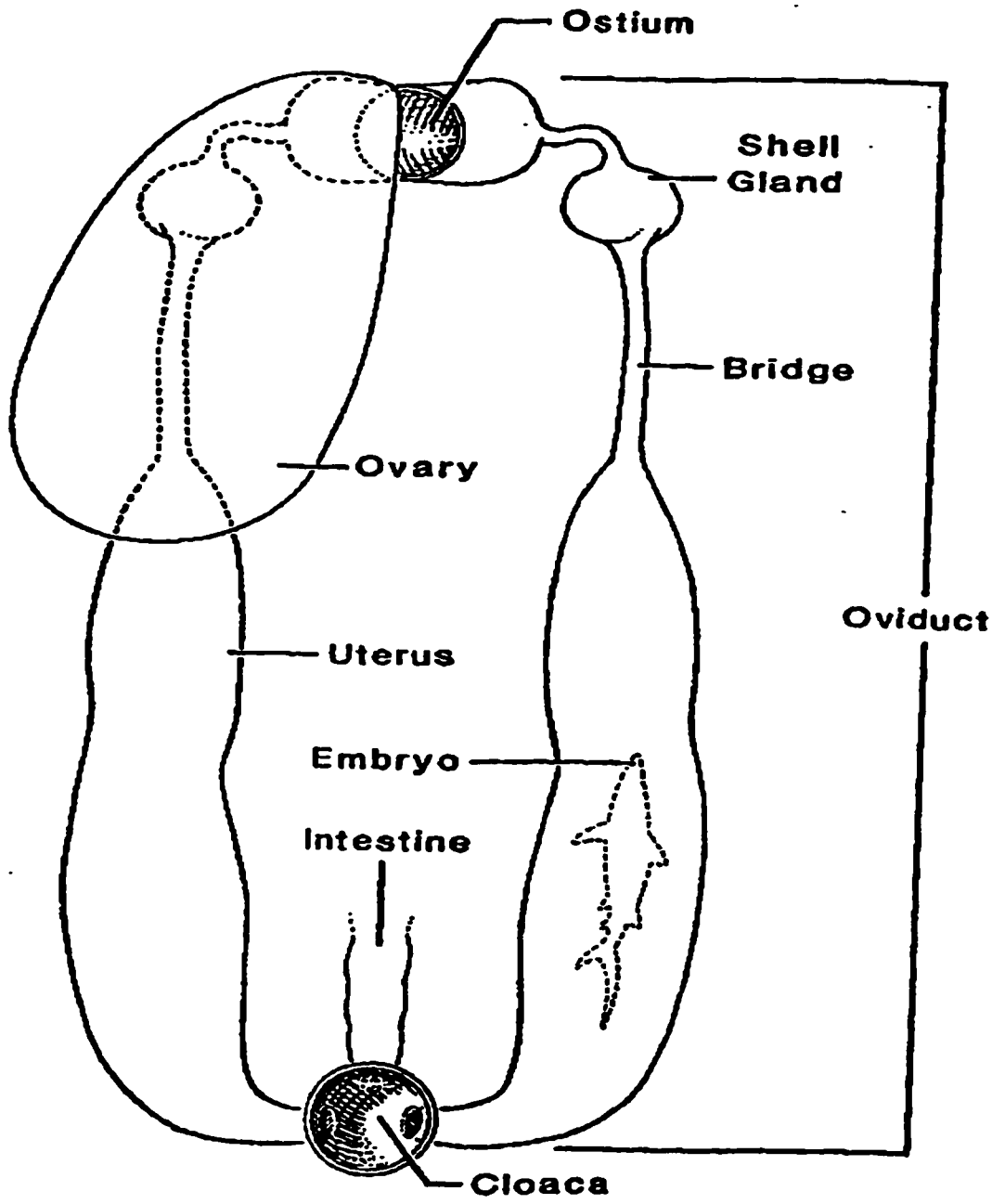

Ostium
Shell
Gland
Bridge
Ovary
Oviduct
Uterus
Embryo
Intestine
Cloaca

Figure 2. The annual reproductive cycle of the female bonnethead shark. Reproductively active tissues highlighted at stage of activity in the female cycle.

Sperm Storage
Oviductal Gland
Yolk Production
Liver
Ovary
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec

Figure 3. Study sampling site on the southwest coast of Florida in Tampa Bay.

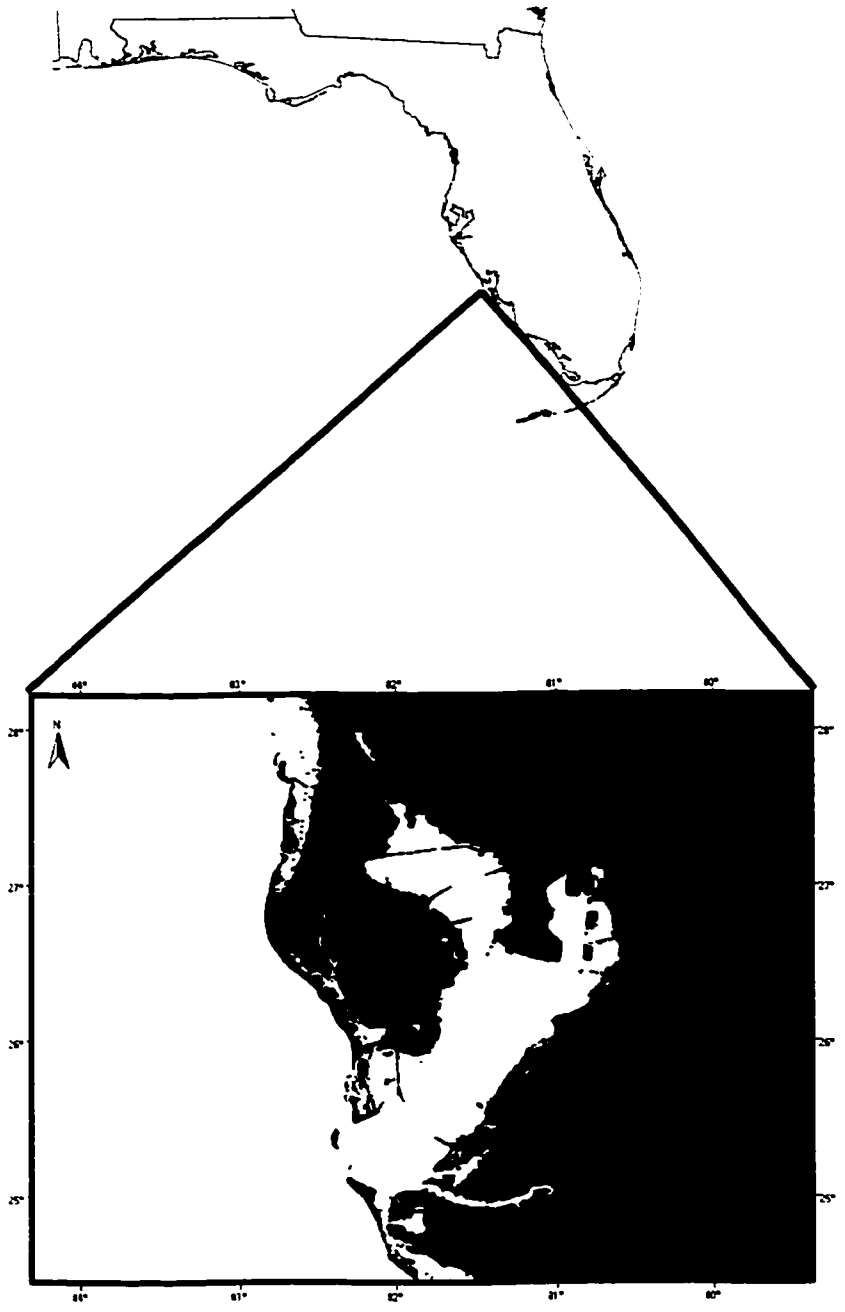


Figure 4. Anti-dasyatid calcitonin antibody dilution curve from indirect ELISA. Inflection point (arrow) at log dilution of 4. Log dilution - logarithm of the antibody dilution factor.

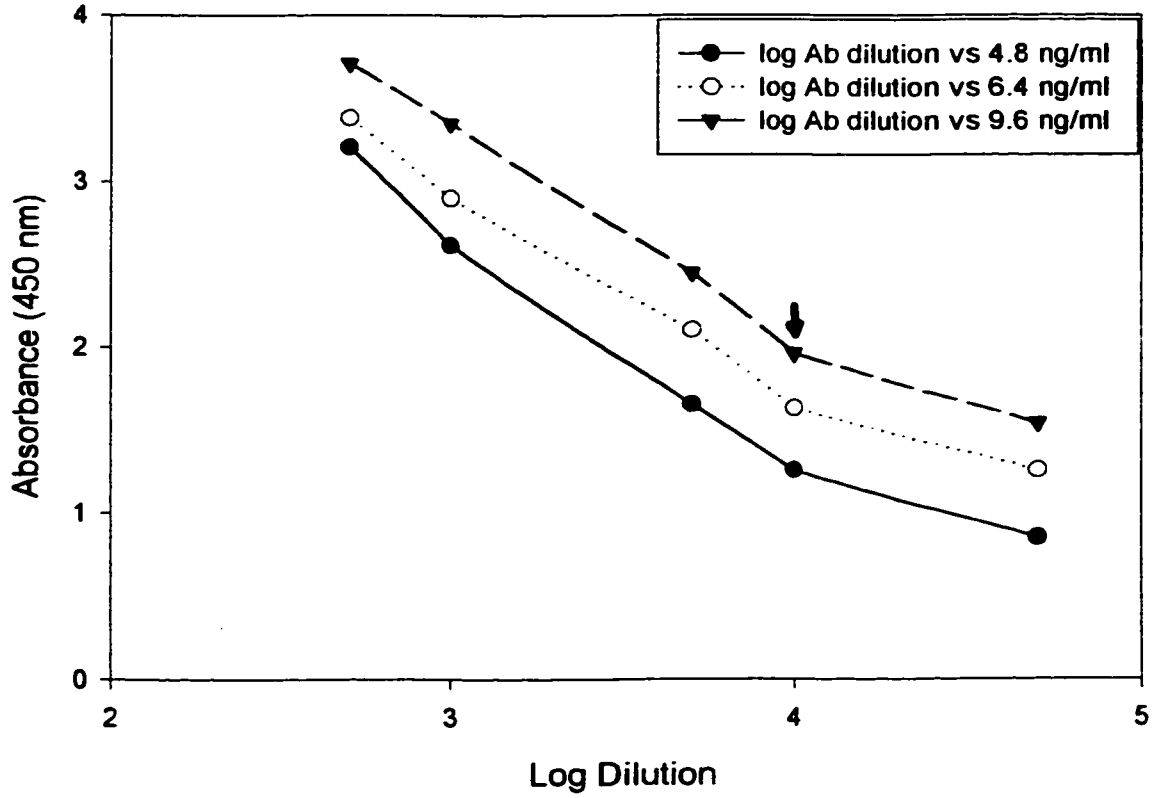

log Ab dilution vs 4.8 ng/ml
log Ab dilution vs 6.4 ng/ml
log Ab dilution vs 9.6 ng/ml
Absorbance (450 nm)
0
1
2
3
4
2
3
4
5
Log Dilution

Figure 5. Dasyatid calcitonin indirect ELISA standard curve. Standard values ranged from 1.6 to 76.8 ng/mL. Coating of 51.2 ng/mL indicated with arrow. Standards were tested against an antibody dilution of 1/10,000.

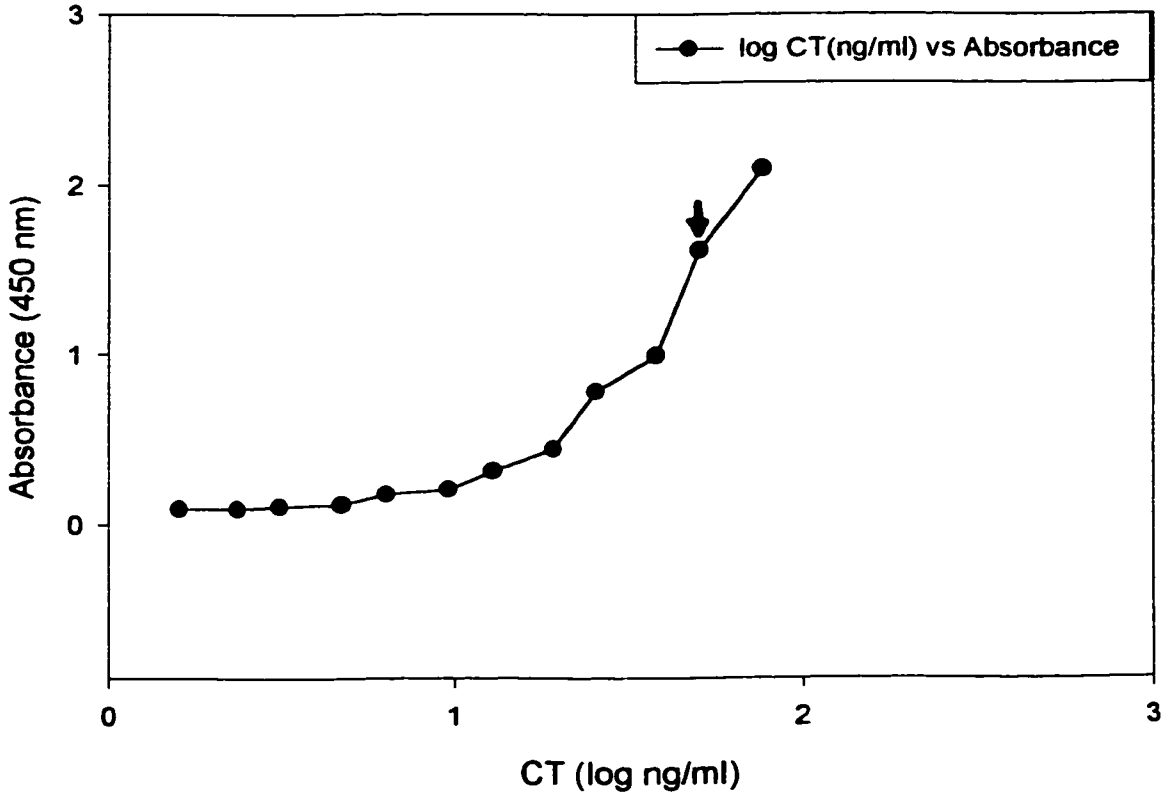

log CT(ng/ml) vs Absorbance
Absorbance (450 nm)
CT (log ng/ml)
0
1
2
3

Figure 6. Dasyatid calcitonin indirect ELISA standard curve and serum dilution curve from late pregnancy, preovulatory and ovulatory stages. The standard and serum curves established did not demonstrate a similar or parallel relationship

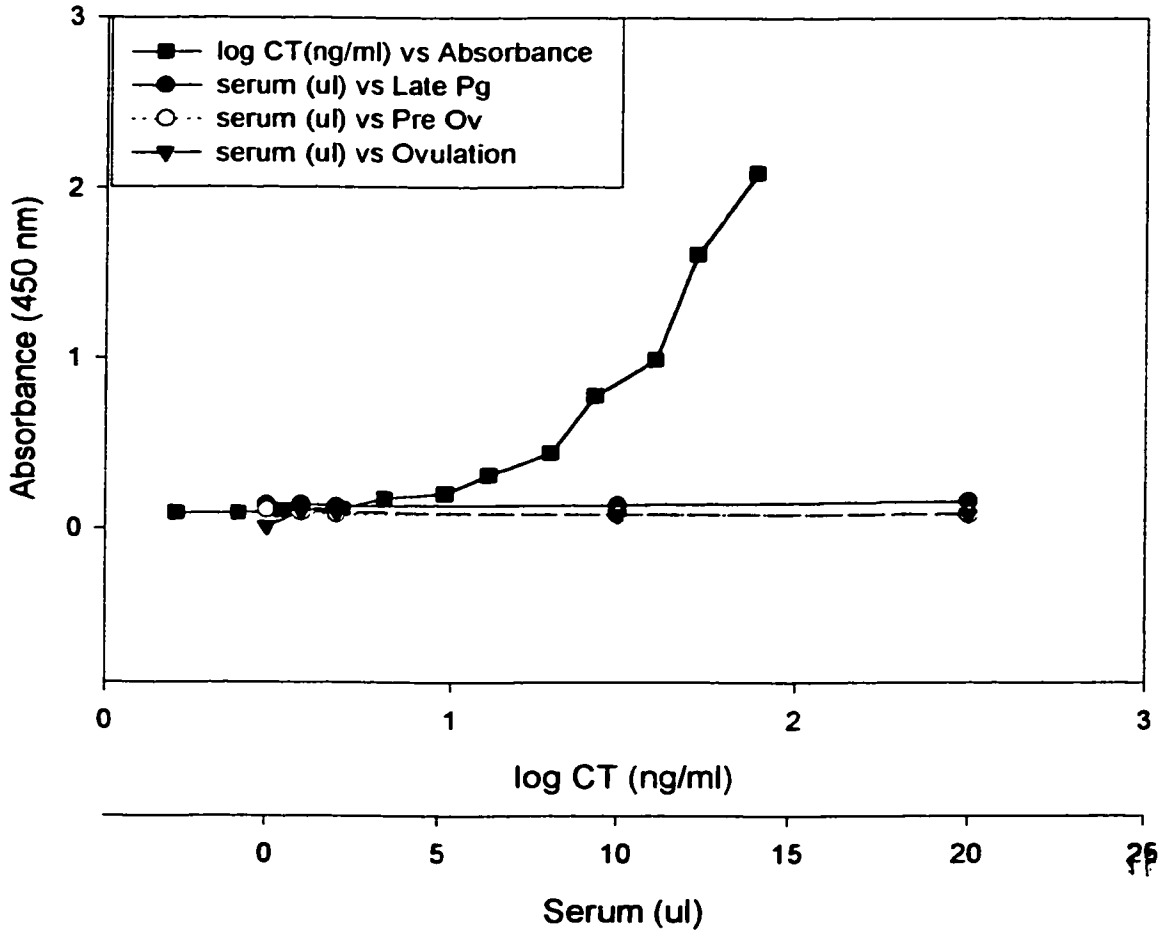

log CT(ng/ml) vs Absorbance
serum (ul) vs Late Pg
serum (ul) vs Pre Ov
serum (ul) vs Ovulation
Absorbance (450 nm)
3
2
1
0
0
1
2
3
log CT (ng/ml)
0
5
10
15
20
25
Serum (ul)

Figure 7. Anti-dasyatid calcitonin antibody dilution curve from competitive inhibition ELISA and serum dilution curve of pooled female bonnethead shark serum. The standard and serum curves established did not demonstrate a similar or parallel relationship.

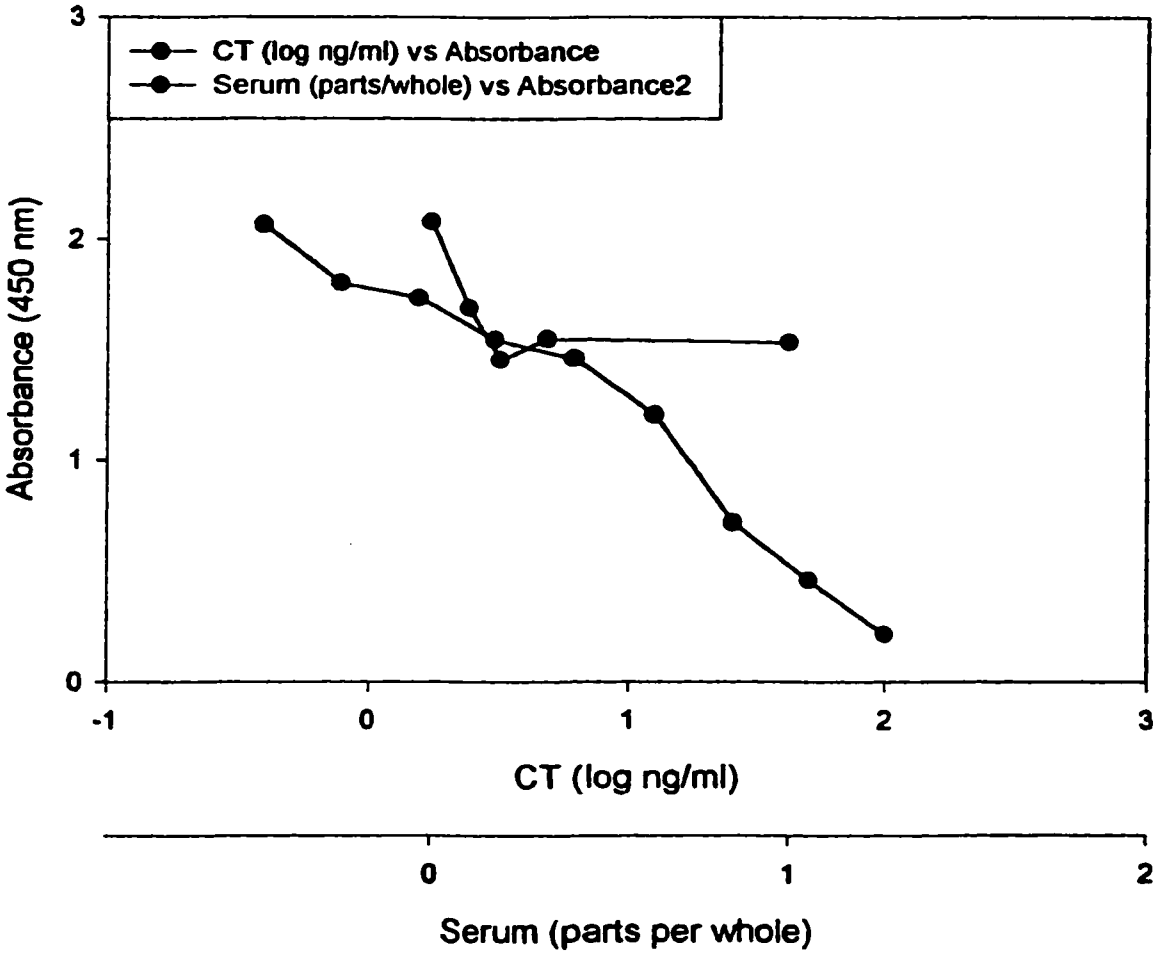

CT (log ng/ml) vs Absorbance
Serum (parts/whole) vs Absorbance2
Absorbance (450 nm)
3
2
1
0
-1
0
1
2
3
CT (log ng/ml)
0
1
2
Serum (parts per whole)

Figure 8. Optimal assay concentration for salmon calcitonin and anti-salmon calcitonin antibody. Wells were coated with serial dilutions of purified salmon calcitonin ranging from 1.6 to 76.8 ng/mL and incubated with various antibody concentrations.

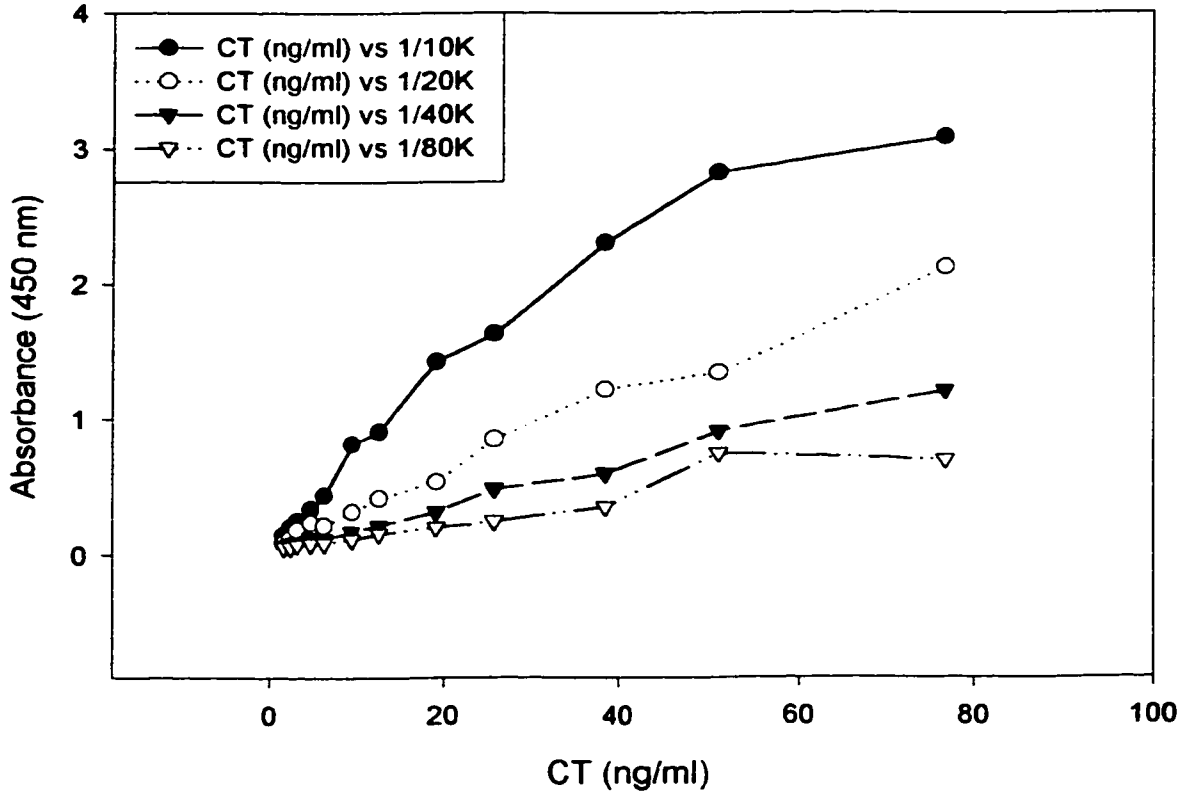
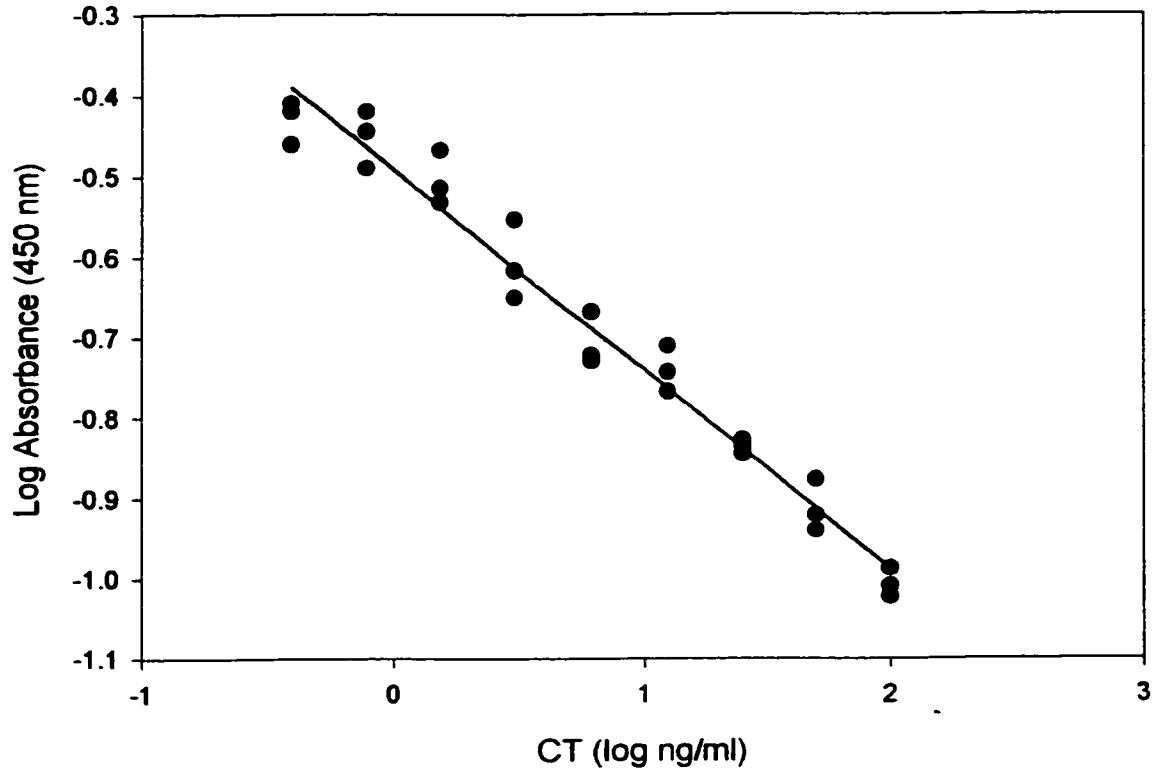

CT (ng/ml) vs 1/10K
CT (ng/ml) vs 1/20K
CT (ng/ml) vs 1/40K
CT (ng/ml) vs 1/80K
Absorbance (450 nm)
0
1
2
3
4
0
20
40
60
80
100
CT (ng/ml)

Figure 9. Log absorbance versus log calcitonin (ng/mL) and log absorbance versus serum parts (mL) graphs demonstrating a similar or parallel dose-response relationship between the standard analyte and unknown (serum) samples.


-0.3
-0.4
-0.5
-0.6
-0.7
-0.8
-0.9
-1.0
-1.1
Log Absorbance (450 nm)
-1
0
1
2
3
CT (log ng/ml)

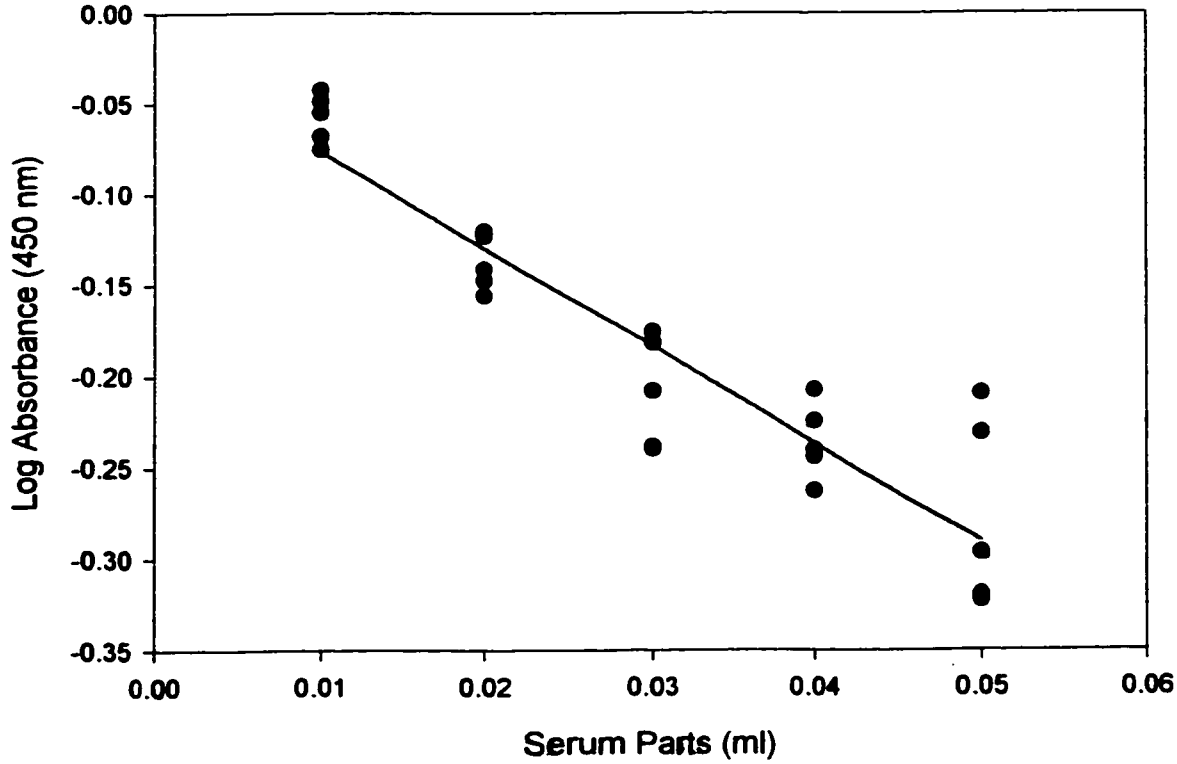

0.00
-0.05
-0.10
-0.15
-0.20
-0.25
-0.30
-0.35
Log Absorbance (450 nm)
0.00
0.01
0.02
0.03
0.04
0.05
0.06
Serum Parts (ml)

Figure 10. Serum calcitonin concentrations in mature female bonnethead sharks from Tampa Bay using a competitive inhibition ELISA. Values were calculated from formula $y = ((-.2722)x + (-.4789))$. Asterisk (*) denotes significant peaks ($P<0.05$, Mann-Whitney U test). N for each stage is noted above.

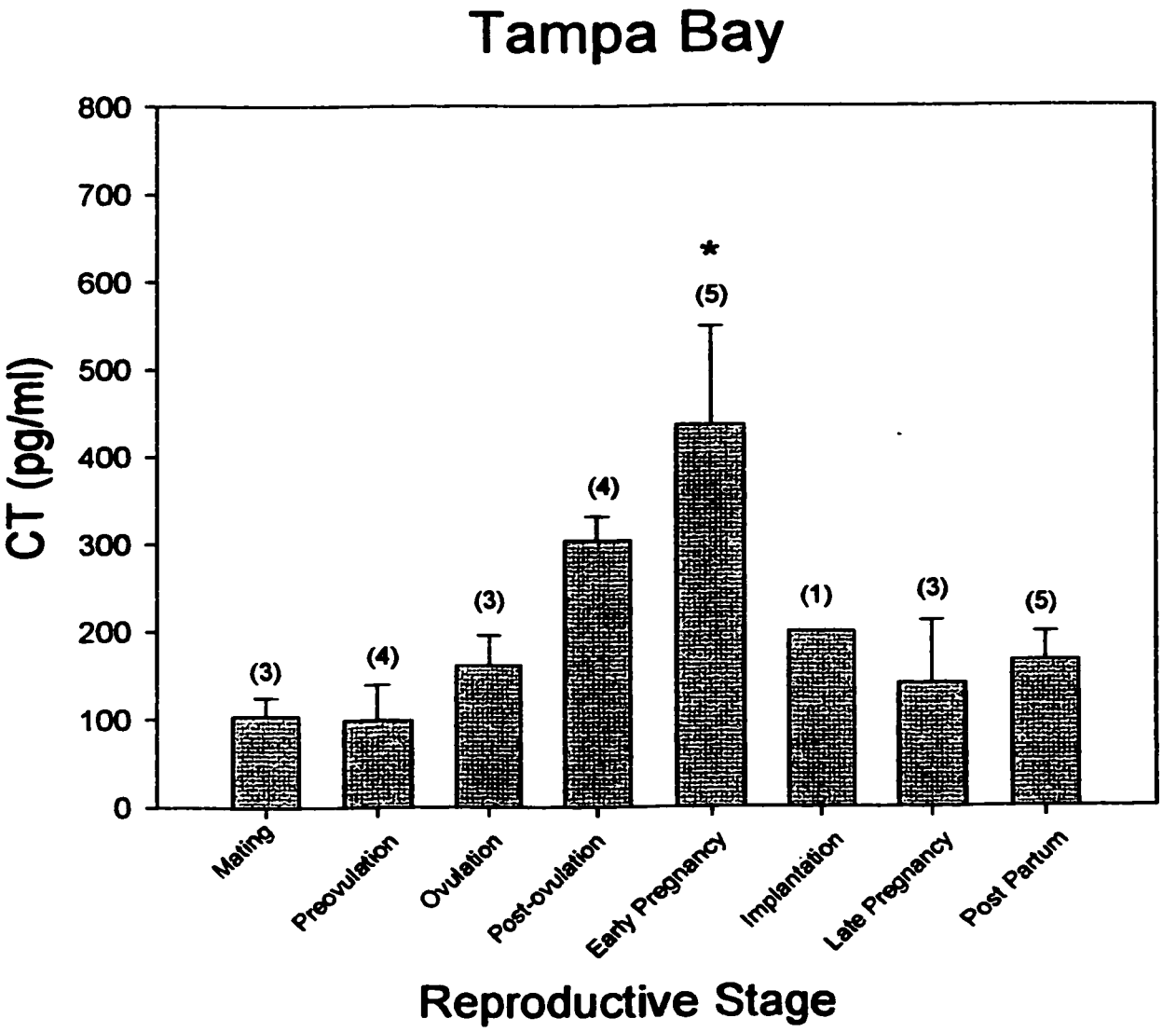

Tampa Bay
800
700
600
500
400
300
200
100
0
CT (pg/ml)
*
(5)
(4)
(3)
(4)
(3)
(1)
(3)
(5)
Mating
Preovulation
Ovulation
Post-ovulation
Early Pregnancy
Implantation
Late Pregnancy
Post Partum
Reproductive Stage

Figure 11. *Sphyrna tiburo* ultimobranchial gland at all levels of experimental control.

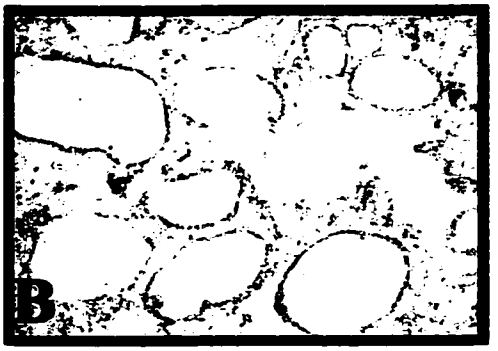
A. HHE stain demonstrating the structure of the gland. The UBG consisted of many follicles (F) lined with parenchymal epithelial cells (E) and contained a granular material in the follicular cavity (G) (20X).

B. Negative control section of the UBG that had been incubated with G-PBS instead of primary antibody during the immunohistochemical procedure (20X).

C. Second negative control section of the UBG that had been incubated with pretreated primary antibody (20X).

D. Positively treated section of the UBG that had been incubated with a primary antibody dilution of 1/10,000 (salmon calcitonin). Strong immunoreactivity was evident in the parenchymal epithelial cells (arrow) (20X).


E
F
G


B


C


D

Figure 12. Female bonnethead shark uterus over the reproductive cycle.

A. HHE stain demonstrating uterine structure during **mating** stage of cycle (10X).
B. IHC stain of mating uterus. No calcitonin immunoreactivity evident (20X).

C. HHE stain demonstrating uterine structure during **preovulatory** stage of cycle (20X).
D. IHC stain of preovulatory uterus. No calcitonin immunoreactivity evident (20X).

E. HHE stain demonstrating uterine structure during **ovulatory** stage of cycle (20X).
F. IHC stain of ovulatory uterus. No calcitonin immunoreactivity evident (20X).

G. HHE stain demonstrating uterine structure during **postovulatory** stage of cycle (20X).
H. IHC stain of postovulatory uterus. No calcitonin immunoreactivity evident (40X).

I. HHE stain demonstrating uterine structure during **early pregnancy** stage of cycle (20X).
J. IHC stain of early pregnancy uterus. No calcitonin immunoreactivity evident (10X).

K. HHE stain demonstrating uterine structure during **late pregnancy** stage of cycle (20X).
L. IHC stain of late pregnancy uterus. No calcitonin immunoreactivity evident (40X).

M. HHE stain demonstrating uterine structure during **post partum** stage of cycle (20X).
N. IHC stain of post partum uterus. No calcitonin immunoreactivity evident (40X).

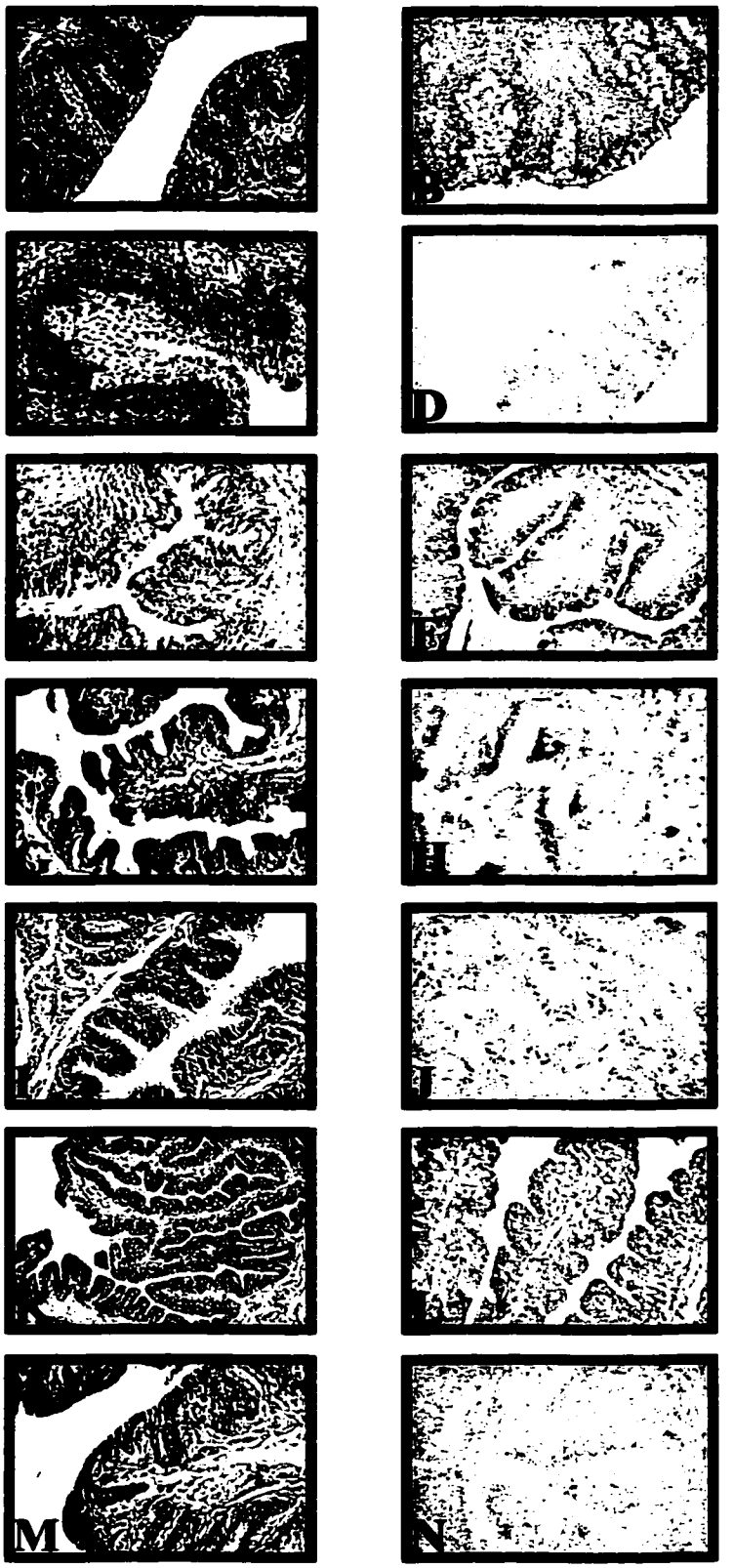
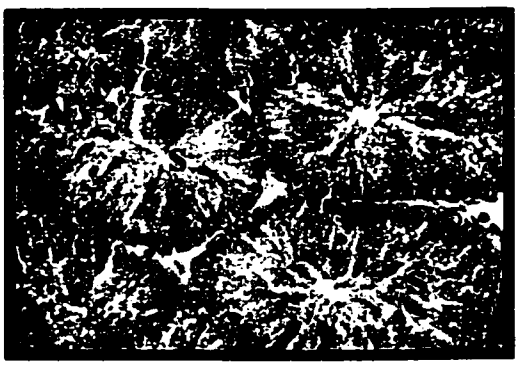

M
N

Figure 13. Reproductively active tissues during the early stages of the reproductive cycle.

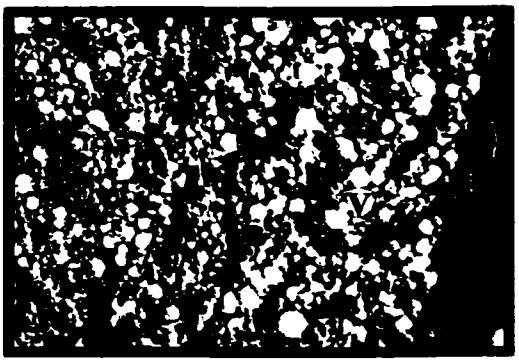
A. HHE stain of the oviducal gland during the mating stage of reproduction. The section demonstrated secretory tubules (S) surrounded by a cuboidal epithelial layer (E) (20X).
B. IHC stain demonstrated no calcitonin immunoreactivity was evident within the oviducal gland secretory tubules (20X).

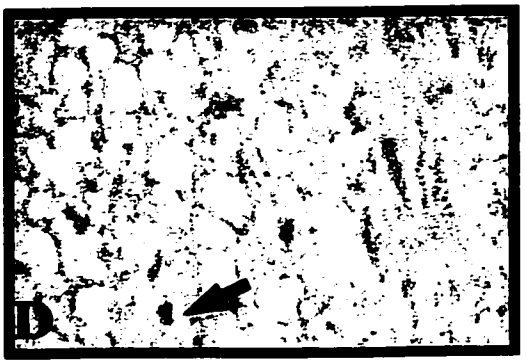
C. HHE stain of adult female bonnethead shark liver during the preovulatory stage of reproduction. The liver was highly vascularized and made up of many large fat vacuoles (V) (20X).
D. IHC stain of the liver. No immunoreactive calcitonin was evident. Melanocytes (pigment producing cells) were seen throughout the tissue (arrow) (40X).

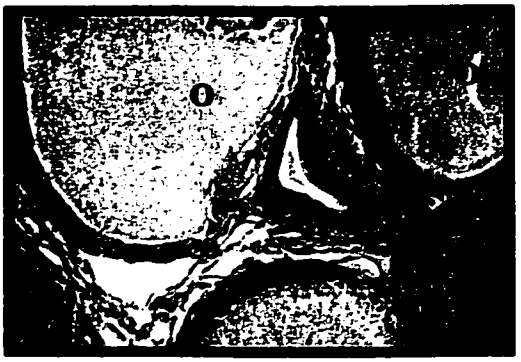
E. HHE stain of the ovary during the preovulatory stage of reproduction. The ovary consisted of many follicles containing oocytes (O) of various sizes embedded within loose, connective tissue (C) (20X).
F. IHC stain of the ovary. No immunoreactive calcitonin was evident (20X).




B




D


O


F

Figure 14. Ultimobranchial gland in the bonnethead shark embryo.

A. HHE stain of the embryonic UBG having an identical location and structure as the adult UBG. The UBG follicles (arrow) were found embedded in the skeletal muscle and cartilage of the pericardial wall. The follicles were lined with epithelial cells and contained granular material in the lumen (10X).

B. IHC stain indicated there was no calcitonin bioactivity at this early stage of development (10X).

Figure 15. Bonnethead shark embryonic tissues involved in gas exchange.

A. HHE stain of the umbilical cord and appendiculae. The umbilical cord consisted of a vein (V) and an artery (A) surrounded by a sheath of connective tissue from which the appendiculae emerged. The appendiculae were lined with columnar epithelial cells (E) (20X).
B. IHC stain of the umbilical cord and appendiculae indicated no immunoreactive calcitonin was present (20X).

C. HHE stain of an internal gill filament of the bonnethead shark embryo. The gill filament held many individual lamellae (L), which consisted of a double sheet of epithelial cells overlying a basement membrane surrounding blood channels (arrow) within the lamella (20X).
D. IHC stain of the gill filament did not indicate immunoreactive calcitonin in the embryonic gill (40X).

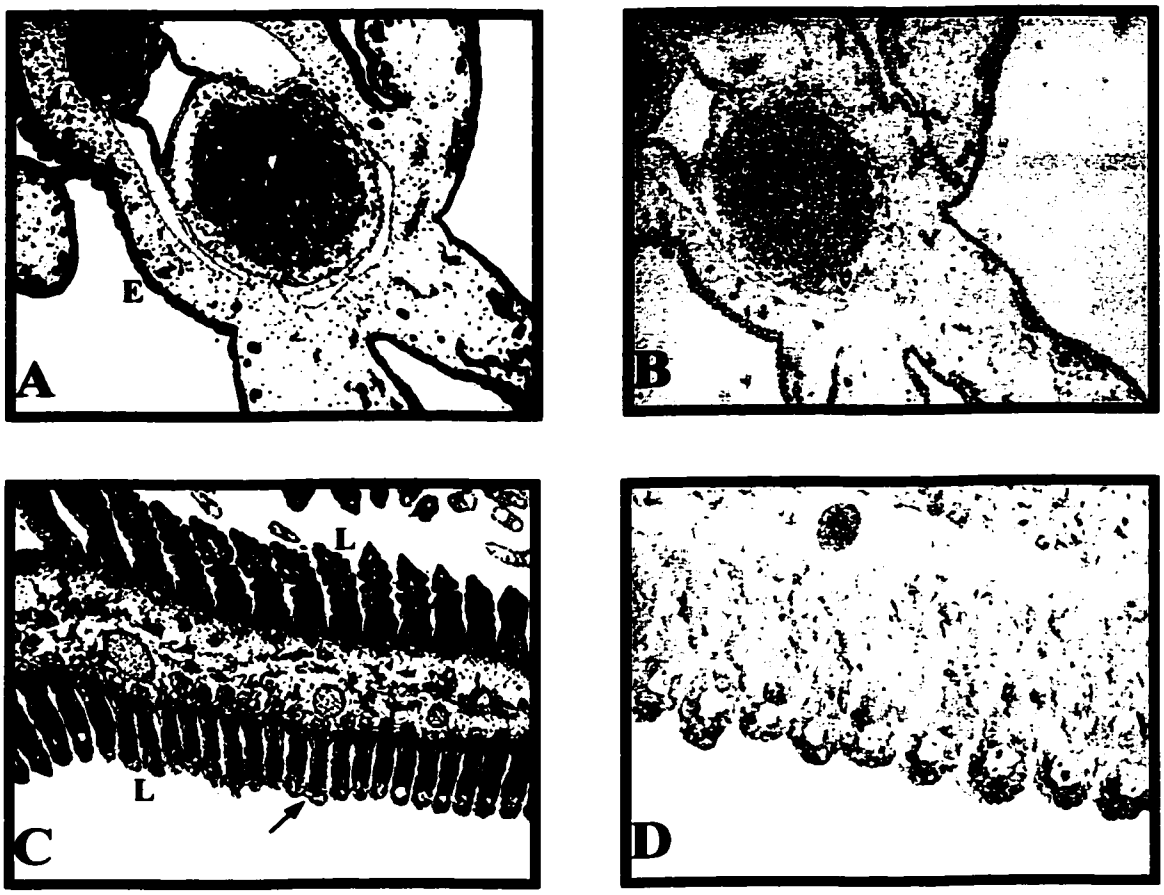

E
A
B
L
L
C
D

Figure 16. Thyroid gland from the bonnethead shark embryo.

A. HHE stain of the thyroid. The thyroid consisted of many lobular follicles (F) lined with columnar to cuboidal epithelial cells (E) surrounding a lumen into which a colloid substance was secreted (10X).

B. IHC stain indicated no immunoreactive calcitonin was evident in the embryonic thyroid.


F
F
A

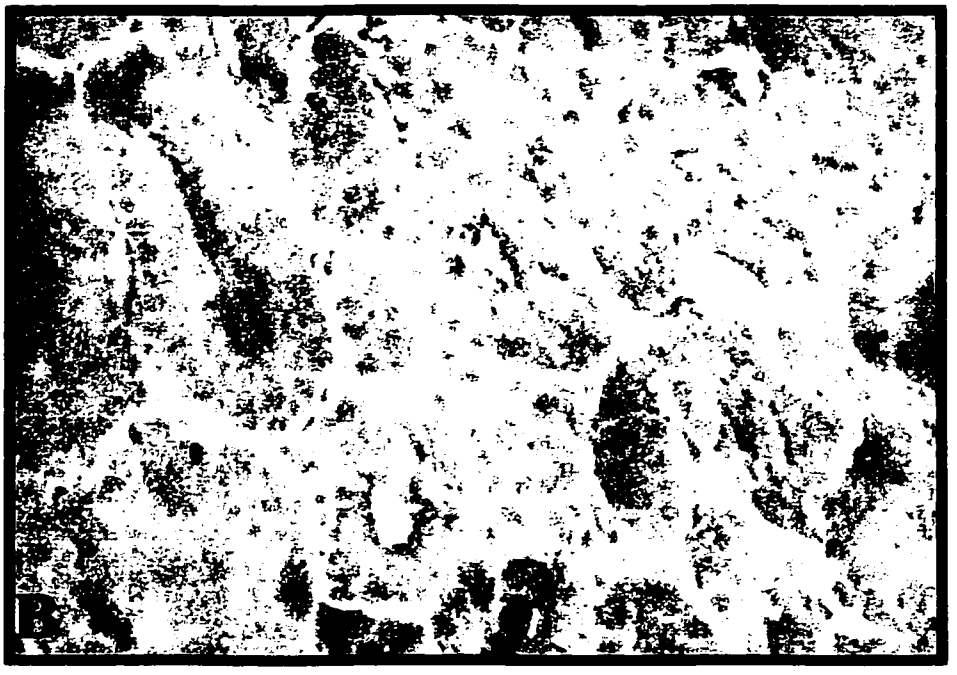
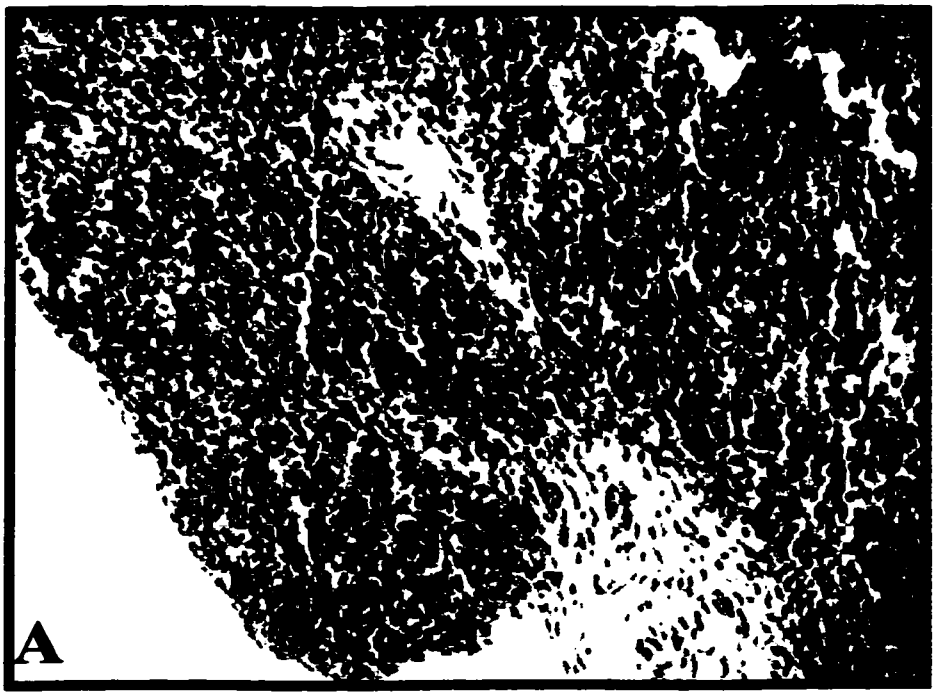


Figure 17. Spleen from the bonnethead shark embryo.

A. HHE stain of the spleen. The spleen was highly vascularized, consisting of masses of red and white pulp containing erythrocytes, lymphocytes, and plasma cells (20X).

B. IHC stain indicated no immunoreactive calcitonin was evident in the embryonic spleen (40X).


A

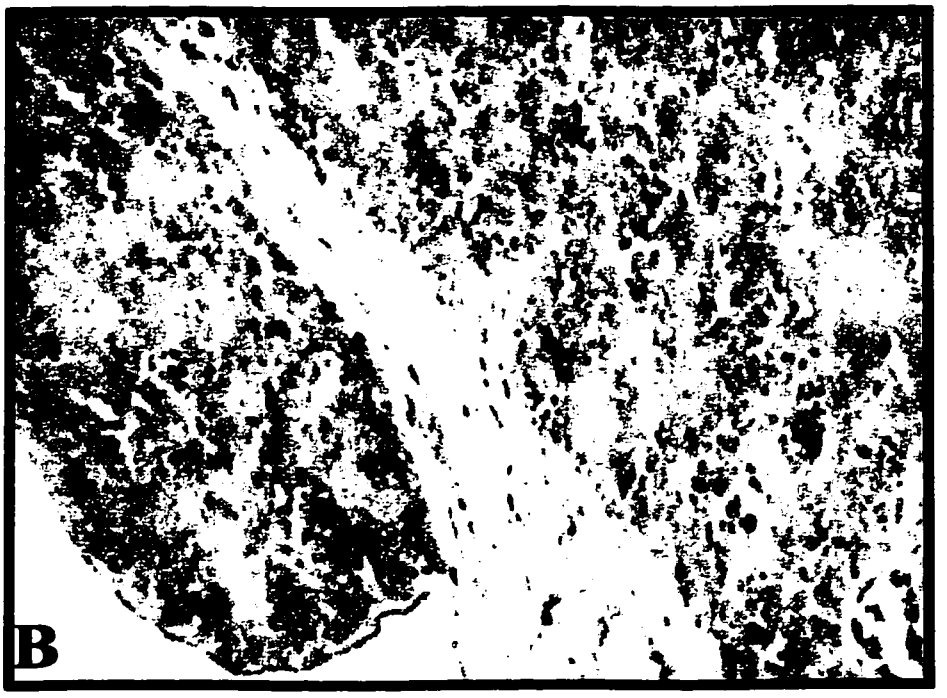

B

Figure 18. Renal complex of the bonnethead shark embryo.

A. HHE stain of the kidney and interrenal gland. The section of kidney shown (K) consisted of "bundle zone" kidney (Hamlett, 1999) – tightly packed tubules. These tubules consisted of columnar epithelium surrounding the tubule lumen into which they drained. The interrenal tissue (I) consisted of tightly packed cells embedded within the kidney tissue (20X).

B. IHC stain indicated no immunoreactive calcitonin was present in the embryonic renal complex (40X).

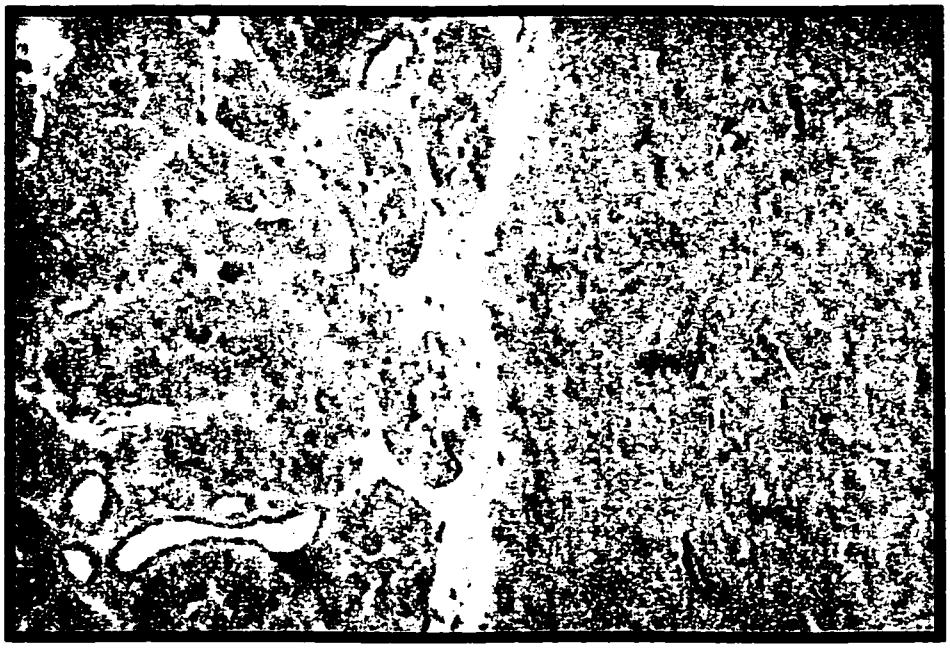


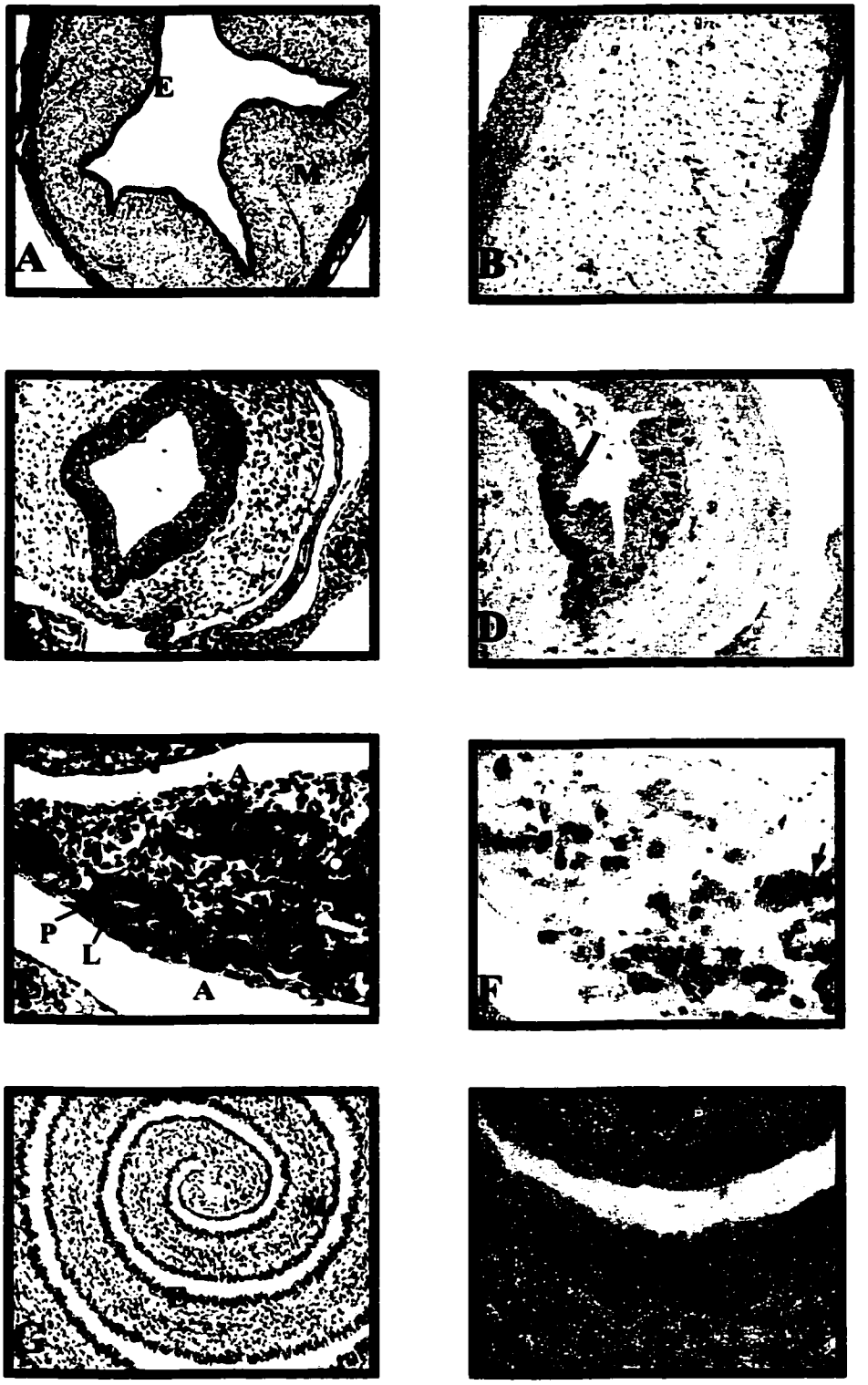
Figure 19. Gastrointestinal tract and pancreas of the bonnethead shark embryo.

A. HHE stain of the stomach. The stomach had columnar epithelial mucosa cells (E) lining the lumen of the gut. The outer border of the stomach was made up of connective tissue and muscle layers (M) (20X).
B. IHC stain of the stomach did not indicate calcitonin immunoreactivity (40X).

C. HHE stain of the duodenum. The duodenum had a similar structure to that of the stomach, however possessed a thicker epithelial mucosa layer (E) (20X).
D. IHC stain of the duodenum of the early pregnancy bonnethead shark embryo indicated calcitonin bioactivity (arrow) within the secretory epithelial cells that lined the duodenal lumen (20X).

E. HHE stain of the pancreas. The alveoli (A) or exocrine ducts of the pancreas were embedded within vascular tissue (V). Each alveoli was made up of large pyramidal cells (P) surrounding a central lumen (L) (20X).
F. IHC stain of the early pregnancy embryo pancreas indicated a positive reaction for calcitonin immunoreactivity (arrow) (20X). The staining appeared to occur in both endocrine and exocrine tissues.

G. HHE stain of the intestine. The scroll valve type of intestine in the bonnethead shark was evident in cross section. Layers of the scroll were lined with columnar epithelial cells (E) overlying a muscle layer (M) (20X).
H. IHC stain of the intestine indicated no calcitonin immunoreactivity was evident (40X).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